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13. ABSTRACT *(Maximum 200 Words)*

The link between diet and breast cancer has been long postulated and recently, extensively investigated. Identifying a molecular mechanism linking diet and breast cancer, however, has remained elusive. Recently, we identified an isoform of the cellular receptor called the peroxisomal proliferator-activated receptor gamma (PPARγ) in human breast cancer cell lines. Activation of PPAR can alternatively lead to tumor induction or differentiation into a more benign state, depending on the tissue and the isoform expressed. We and others have demonstrated that human breast cancer cell lines express PPARγ and that individual fatty acids are capable binding to and functioning as selective agonist or antagonist of PPAR. Furthermore, we present evidence that the signal transduction of PPAR can be mediated by the presence of the estrogen receptor. We have verified the fidelity of our transcriptional reporter system using antisense expression vectors in transient transfection analysis and begun to examine the regulation of expression of this gene in breast cancer cells. These studies may lead to better understanding of the risk of specific dietary components as fatty acids can indeed function as hormones and further investigation could lead to changes in dietary guidelines. The implications of these studies could have a profound impact on both prevention and management of this devastating disease.

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Table of Contents

INTRODUCTION

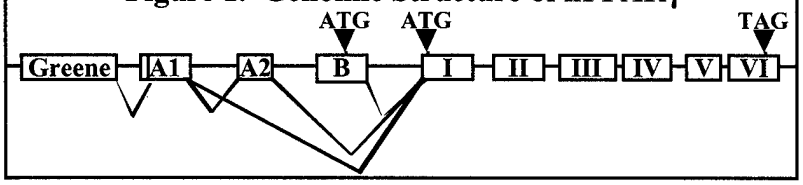
The incidence of breast cancer has steadily risen over the past 5 decades and is now one of the leading causes of cancer related deaths among American women [1]. While intensively investigated, the cause of this rise remains unexplained. Not all countries have experienced this rise in breast cancer risks but these differences cannot be accounted for by genetic factors [2]. Although epidemiological analysis suggests a correlation between high fat diets and breast cancer, much controversy remains. Animal studies, by contrast, have provided convincing evidence of a correlation between dietary fats, types of fats ingested and mammary tumors. In our efforts to determine if a molecular mechanism exists to link dietary fats to breast cancer incidence, we cloned the peroxisome proliferator-activated receptor gamma (PPARγ) from several breast cancer cell lines [3]. We have further demonstrated that individual fatty acids are able to positively or negatively mediate transactivation of PPAR in breast cancer cell lines [4]. PPARγ is a nuclear receptor with wide–ranging roles in tumor and normal development [5], atherosclerosis [6], angiogenesis [7] and adipocyte differentiation [8]. As differentiation was initially thought to be a universal mechanism of PPARγ transactivation our demonstration of a functional PPAR response in human breast cancer cells [3] generated a great deal of interests in using PPARγ-specific ligands for the treatment of breast cancer. Various ligands have been reported to induce apoptosis [9,10], initiate cell cycle withdrawal [9,11] and mediate the transcription of target genes consistent with a more differentiated, less malignant phenotype [12]. The goal of this work was to determine what forms of PPAR and RXR are expressed in breast cancer cells and determine whether transactivation by dietary fatty acids might be important in the etiology of breast cancer. To this end, we have determined the isoforms expressed by molecular cloning, examined the regulation of expression and investigated the molecular mechanism of transcriptional activation of PPAR using cell culture systems. What we have discovered is a complex and important role for PPAR in human breast cancer cells. The data presented here has already lead several groups to examine new approaches in the treatment of breast cancers. Furthermore, these data could lead to new targets in the prevention of breast cancer through the development of novel therapeutic interventions and revised dietary guidelines for women at risk of breast cancer as well as breast cancer survivors.

Body

Research Accomplishments:

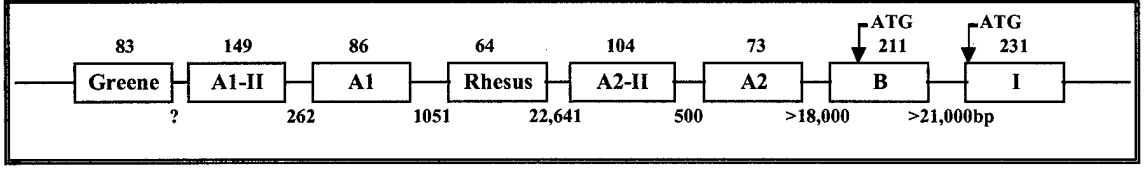
The peroxisome proliferator-activated receptor is a member of the nuclear receptor superfamily of transcription factors. PPAR has three known members, α, β (also termed δ) and γ (NRC1, C2 and C3, respectively) [13] and within gamma, 3 more subtypes have been identified, γ1, γ2 and γ3. Using a mouse cDNA we originally determined that PPAR is expressed in both MCF-7 and T-47-D human breast cancer cells by Northern blot analysis. To confirm this observation and to identify the form(s) of PPAR expressed a cDNA library was constructed from MCF-7 cells and screened with both PPAR and RXR probes. These were designed against the DNA binding domain of the receptors, the most highly conserved portion of the gene, to identify all forms present. The isolated C1 clone was clearly PPARγ, containing the ATG start site at base +173 (Accession # L40904) and the stop sign at base 1607 which encodes the full open reading frame of the 478 amino acid protein. Our sequence diverged from that reported by Greene and co-workers [14] immediately 5' of the start site of translation. An additional 74 bases were inserted between the untranslated exon A1 [15] and the ATG-containing exon (exon I). This was later described as exon A2 and reported to itself be associated with a promoter element termed PPARγ3 [16]. As seen in figure 1, exon A2 is either a splice variant of γ1 expression or can be the first untranslated exon and may be associated with unique promoter elements. PPARγ1 and 3 code for identical proteins while γ2 contains an additional 28 N'-terminal amino acids. The functional significance of these differences remain unclear. Nonetheless, none of the cDNA's we have cloned and none of the RT-PCR products we have been able to amplify from mRNA isolated from breast cancer cells contain the Greene sequence or Exon B. For these reasons, we believe the only protein expressed in these cells is PPARγ1. The regulation of expression, however, is much more complex.

Figure 1. Genomic Structure of hPPARγ



Since the genomic structure and thus the regulation of PPAR is complex, we first sought to characterize the promoter structure and determine if other forms of PPAR are expressed in MDA-MB-231 cells which have not been previously reported. To this end we have performed 5' RACE (rapid amplification of cDNA ends) to delineate the start site of transcription. Thus far we have isolated 4 clones which are depicted in figure 2. In some mRNAs exon A1 is fused to A2, then exon I, similar to previous reports. Other clone contains A2 fused to exon I but also contains more than 180 bases, exon A2-II, of the 5' flanking sequence of A2 previously reported to be γ3 promoter sequence. Seventy-four bases are missing from this clone indicating that this is intronic sequence. Analysis of the genomic sequence indicates that the regions flanking this 500-base intron are consensus slice acceptor sites. The third clone contains an exon reported only in Rhesus monkeys (AF033342) fused on to the common slice acceptor site associated with A2 and then onto exon I. The human sequence we have cloned is 96% identical to that of the Rhesus Macaque. The fourth clone we isolated is unique and has

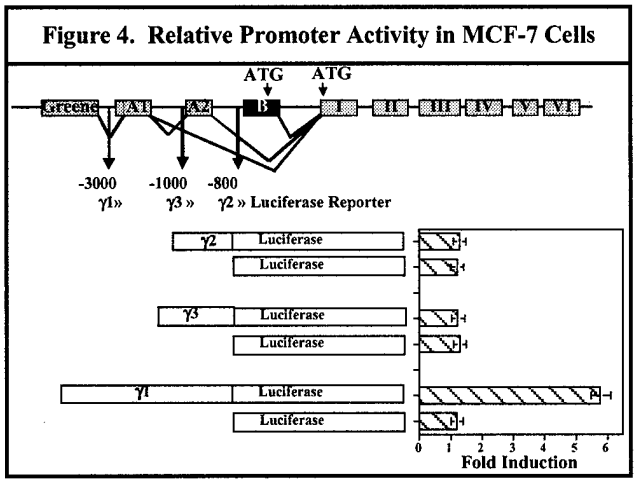
Figure 2. Promoter Structure of PPARg with Exon (above) and Intronic (below) Sizes.



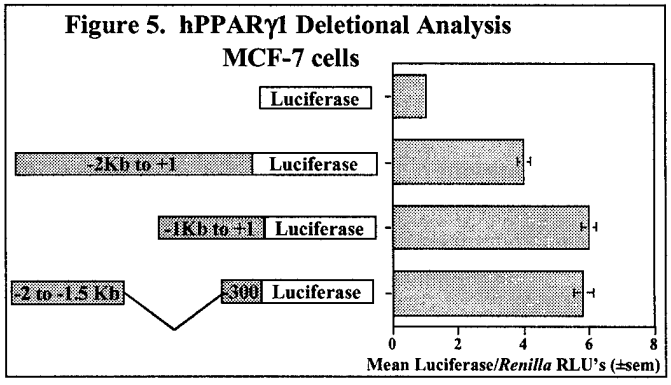
not been previously reported to Genebank, exon A1-II. Genomic structure analysis indicates that this exon lies immediately upstream of exon A1 and is spliced onto A1 and then A2.

To assess the regulation of PPAR in human breast cancer cells we have performed RNAse protection assays and promoter analysis. First we constructed plasmid using the unique 5' regions of each of the 4 clones we isolated from RACE for *in vitro* transcription (Ambio, Austin TX). These were used to prepare RNAse probes for each using mRNA from MCF-7 cells. These data indicate that the only significant promoter appears to be associated with exon A1 (figure 3). As an independent assessment of promoter usage, we have obtained three genomic reporter constructs that drive the tissue-specific expression of PPAR in adipocytes and hepatocytes specific for γ1, 2 and 3 [15,16]. These contain 3000 bp upstream of A1, 1000 bp upstream of B and 800 bp upstream of A2, respectively, driving the expression of luciferase (a kind gift from Dr. Johan Auwerx, Institut de Genetique et Biologie Moleculaire et Cellulaire, Illkirch, France). These were transfected into both MCF-7 and MDA-MB-231 cells and relative luciferase activity compared to control plasmids containing the basic luciferase vector (Promega) lacking the 5' flanking sequences. The data shown in figure 5 is typical of what is seen in both cell lines. These data indicate that the 3000 bp flanking the 5' of exon A1 and not the elements upstream of B or A2, contains *cis*-regulatory elements capable of mediating basal expression of PPAR in both MCF-7 and MDA-MB-231 (figure 4). Both analysis indicate that promoter elements associated with exon A1 are the only significant regulators of PPARγ expression in these cells.

Figure 3. RACE Clones Isolated from MDA-MB-231 and MCF-7 Cells			RNAse Protection Analysis in MCF-7 cells
Exon A1	Exon A2	Exon I	98.6%
5' flanking A2	Exon A2	Exon I	0%
AF033343	Exon A2	Exon I	0%
Unreported	Exon A2	Exon I	1.4%



To begin to examine the sequences that confer basal expression of PPARγ1 in these cells, we have constructed deletion mutants of the 3 kb sequence flanking the 5' of the transcriptional start site and assessed their ability to mediate transcription of a reporter gene. Deletion mutations were created by restriction digestion and religation and all structures were confirmed by sequence analysis. All were compared to the null vector containing the luciferase reporter but lacking the 5' flanking sequence. The entire 3 kb fragment confers basal transcriptional regulation of PPARγ1 and removal of the distal 1000 bp does not alter reporter activity. Removal of an additional 1000 bases does not have a significant effect on basal expression, suggesting that the regulation of basal transcription in these cells is mediated by the proximal 1000bp. To further asses this hypothesis, an additional mutation was constructed lacking the proximal 1000 bp but containing the distal 2 kb removed in the prior construct (figure 5). This sequence appears to have no positive regulatory elements. Furthermore, the distal 500 bp between –2000 and –1500 fused to the proximal 300 bases contains the same reporter activity as that seen in the

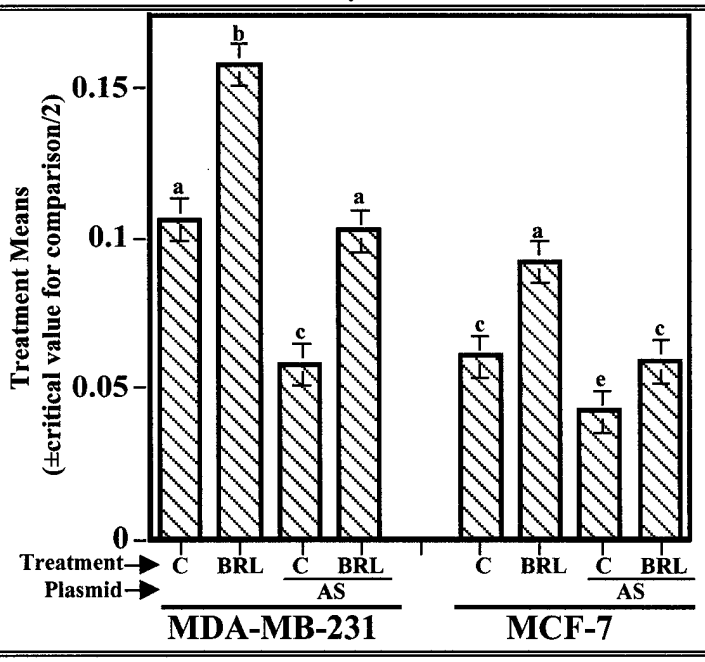


proximal 1000 bp. This suggests that no negative regulatory elements are present between –1500 and –2000 and that the positive basal regulation of PPARγ1 in these cells is contained in the proximal 300 bases of the promoter.

In contrast to the complex pattern of PPAR expression, a single cDNA species of RXR was isolated from both the MCF-7 and the MDA-MB-231 libraries. Despite using a degenerate probe that would have hybridized to all forms of RXR under the conditions used in Southern blot analysis, the only cDNA identified was RXRα. A full-length cDNA was isolated and contained no differences from those reported previously.

To examine the transactivation of PPARγ1 in breast cancer cells we have constructed transactional reporters containing peroxisome proliferator response elements (PPRE). These have been used in transient transfection assays to assess the transactivation of the PPAR-RXR complex. We have demonstrated that synthetic peroxisome proliferators increase the activity of transcriptional reporters containing either the peroxisome proliferator response element (PPRE) or the perfect direct repeat (DR-1) response element. Furthermore, we have gone on to demonstrate that individual fatty acids are capable of selectively functioning as agonists or antagonist of PPAR. This observation touches the central questions regarding whether PPAR mediates the effects of individual fatty acids present in diets and alters the incidence or growth rates of breast tumors. These data also indicate that PPAR is constitutively transcribed, translated and transactivated since treatment with ω-3 fatty acids reduced reporter activity to levels below control (see attached manuscript). Furthermore, transactivation of PPAR with fatty acids correlates with proliferation *in vitro*. It is not clear, however, whether this is a direct or indirect effect. To prove that the transcriptional reporter is indeed measuring the transactivation of PPAR we have constructed a PPARγ antisense expression vector in order to block translation of the mRNA and thus reduce the basal levels of the protein. The antisense expression vector codes for an antisense mRNA spanning the sequence between the start of translation at base 173 and base 693 (accession # L40904). As seen in figure 6, the expression of the antisense mRNA selectively inhibited reporter activation while the sense vector had no effect (data not shown). Furthermore, the antisense expression not only inhibited BRL-stimulated transactivation but it also inhibited basal transactivation again indicating that PPAR is constitutively transactivated in these cells. Finally, MCF-7 cells have lower basal and BRL-stimulated reporter activation than do 231 cells. These findings have lead us to speculate that these two lines of breast cancer cells have differential responses to PPAR ligands and may be due to the differences in the expression of other receptors or cofactors.

Figure 6. PPRE-Mediated Transcriptional Activation Is Inhibited by the Expression of PPARγ1 Antisense



Since many breast tumors are initially estrogen receptor (ER) positive and become ER negative and PPAR has been shown to mediated the transcriptional regulation of estrogen target genes, we have sought to determine whether either form of the ER (alpha or beta) participates in the transactivation of PPAR. MDA-MB-231 cells, which do not express ER, were transfected with the PPRE-mediated reporter with (α or β) or without (C) expression vectors for ERα or ERβ. As seen in figure 7, the mere presence of the ER inhibited PPAR transactivation and inhibited transactivation by the synthetic PPAR ligand BRL 48,482 (BRL). Furthermore, this inhibition of PPAR transactivation is independent of ligand as the presence of 17β estradiol (E_2) had no effects on ER inhibition of PPAR. Furthermore, the presence of the pure antiestrogen ICI 182,780 (ICI) was without effect regarding PPAR transactivation (fig 8). To further ensure that these responses are mediated by the ER isoforms, we have demonstrated that cells transfected with alpha or beta responded to estrogen treatment as determined by their ability to mediate the expression of an ERE-reporter. As seen in figure 9, transfection of cells with either expression plasmid renders then estrogen responsive. Furthermore, these responses were completely inhibited in the presence of ICI. Coupled with the observations regarding PPAR mediated transcriptional regulation of ER target genes, these observations suggest that signal cross-talk exists bidirectionally between ER and PPAR and their respective response elements.

Figure 7. PPRE-Mediated Transcriptional Activation in 231 Cells Expressing ERα or ERβ

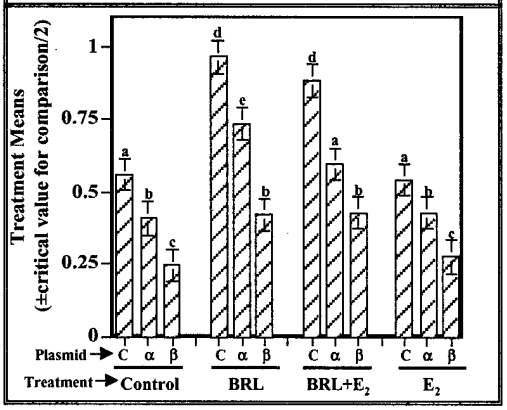


Figure 8. PPRE-Mediated Transcriptional Activation in MDA-MB 231 Cells Expressing ERα or ERβ

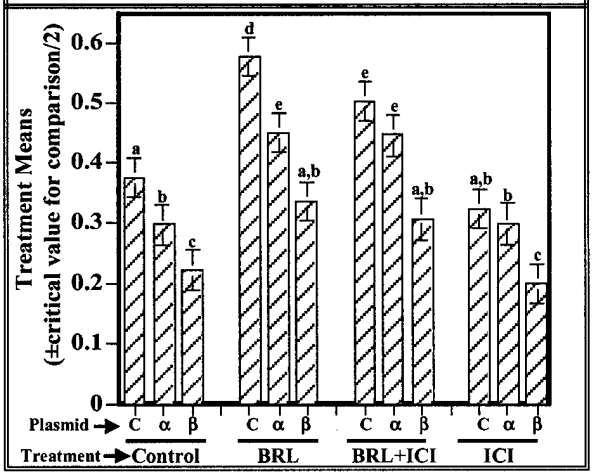
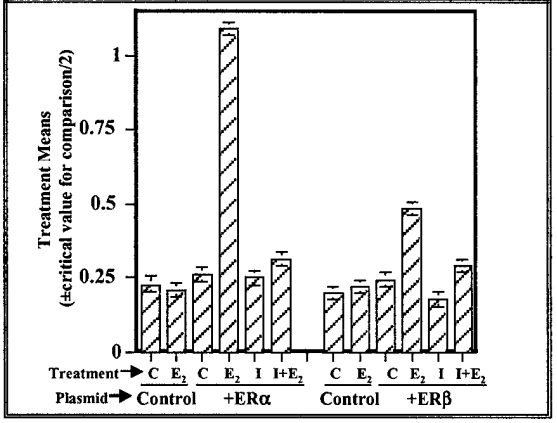
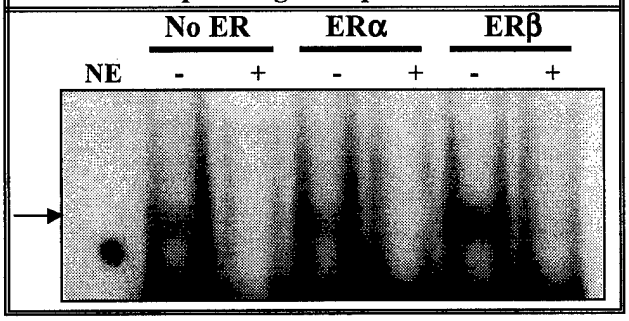


Figure 9. Transfection of MDA-MB-231 Cells with ERα or ERβ Expression Vectors Re-establishes Estrogen Responsiveness.



To examine the mechanism of the signal cross talk we have sought to determine the role of the ER in PPRE binding. Using gel shift analysis we have sought to determine whether the presence of either ERα or ERβ alters the ability of PPAR to recognize and bind to a PPRE. MDA-MB-231 cells were transfected with either an ERα or ERβ expression vector and nuclear proteins isolated form these untransfected cells. As seen in figure 10, the presence of either form to the ER does not prevent PAPAR from binding to the PPRE suggesting that this interaction is not due to competition for DNA binding. To further explore this possibility, we

Figure 10. PPRE Binding in MDA-MB-231 cellsExpressing ERalpha or ERbeta



have gone on to examine the region of the ER necessary for this inhibition of PPAR transactivation. ER, like PPAR and the other nuclear receptors in this family, have domains that function in transactivation, AF1 and AF2, and DNA binding, DBD. Although gel shift analysis suggests that no direct interactions exists between the ER and the PPRE, we have examined which of these domains might be involved it PPAR repression. To this end we have obtained an expression vectors for the wild type ER (a kind Gift from Dr. Benita Katzenellenbogen, University of Illinois) and constructed vectors that will code for proteins lacking the AF1, AF2 or the DNA binding domain. As shown in figure 11, MDA-MB-231 cells were transfected with the PPRE-mediated reporter alone or in conjunction with either the wild type ERα, or mutants lacking one of the three domain as shown. Although the mutants lacking the AF1 and AF2 domains retained their ability to repress PPAR transactivation, deletion of the DBD rendered this construct ineffective. The molecular mechanism of this effect and the role of the DBD in this process are still under investigation. Nevertheless, it is clear that the presence of a functional ER alters these cells ability to respond to PPAR-mediated changes in transcriptional regulation. To further asses the specificity of ER on PPAR suppression we have examined the effects of other nuclear receptors on inhibition of PPAR. We have expressed either the Aryl hydrocarbon receptor or the human androgen receptor in MCF-7 cells and measured PPAR-mediated reporter activation (figure 12). The fact that the expression of the human Androgen receptor nor the human aryl hydrocarbon receptor did not alter PPRE-mediated reporter activation indicates this is a specific action of ER and not due to non-specific squelching. The physiological consequences of the ER effects reported here are also under further investigation. This observation could have important implications for breast cancer treatment as the use of antiestrogen such as Tamoxifen is widely employed. The functional significance of this observation and the molecular mechanisms of this effect are under investigation.

Figure 11. PPRE-Mediated Transcriptional Activation in MDA-MB 231 Cells With and Without Wild-Type ERα and Deletion Mutants

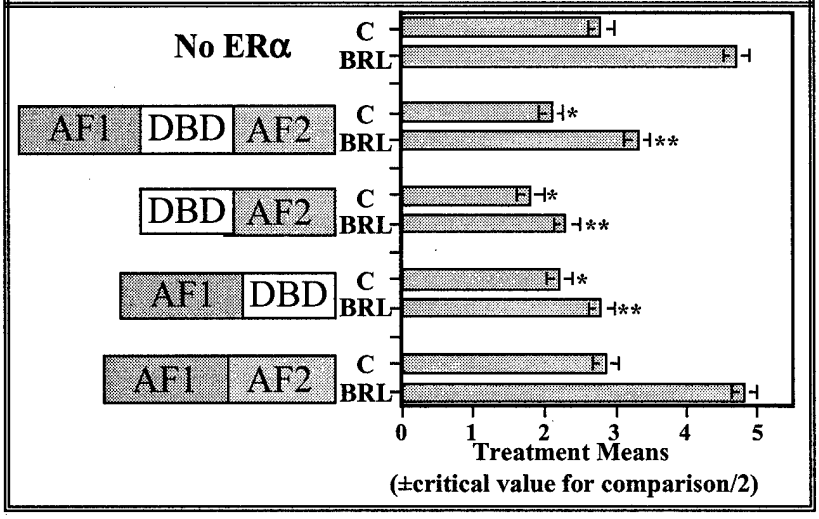
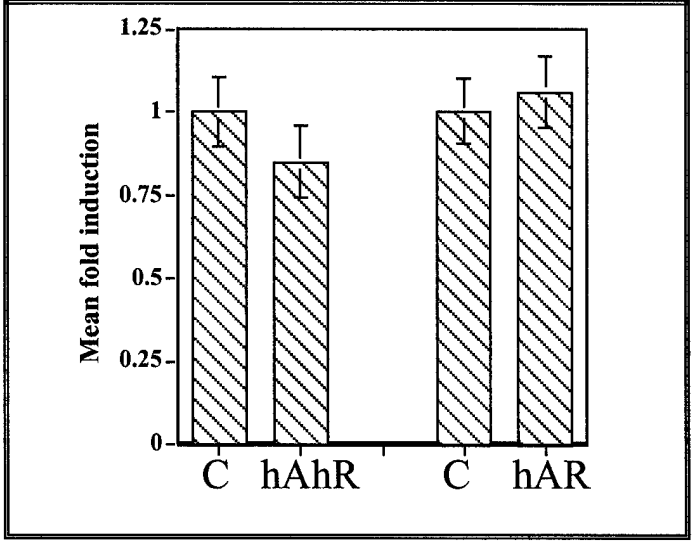
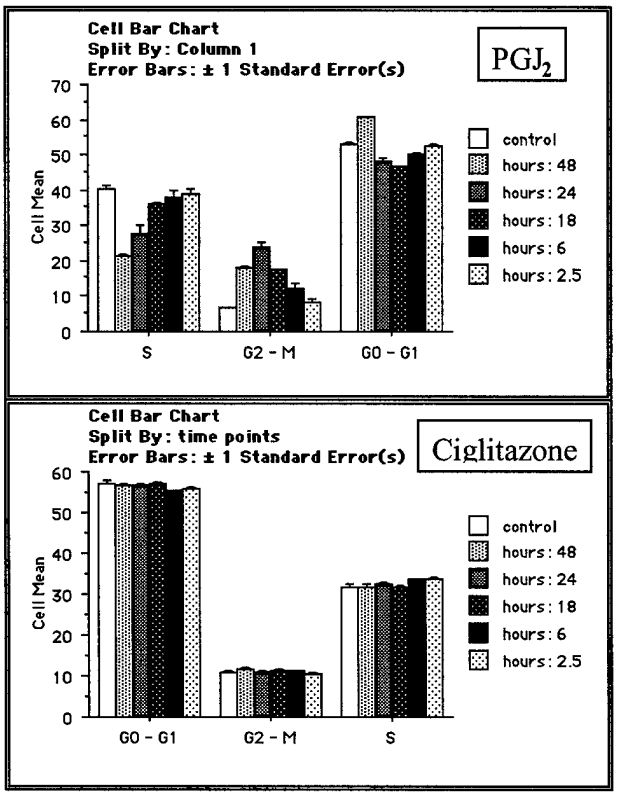


Figure 12. Expression of the Aryl hydrocarbon Receptor or the Androgen Receptor does not Squelch PPAR Transactivation





Data obtained from the activation of PPARγ by fatty acids lead us to postulate that different ligands could alter the receptor structure in ways that result in distinct physiological responses. We have set about to test this concept of SPARM's actions in breast cancer cells. Using 2 distinct PPARγ ligands, Ciglitazone (a synthetic thiazolidinedione) and PGJ_2 we have examined their role in altering cell cycle progression. MCF-7 cells treated with or without each were labeled with propidium iodide and analyzed in the cell sorter. These data demonstrate that Ciglitazone had no effects on cell cycle dependence. Conversely, PGJ_2 resulted in a time-depended decrease in the number of cells replicating DNA (S), an increase in the percentage of cells that were arrested at the point of cell division (G_2-M) and an increase in the percentage of cells in cell cycle arrest (G_0- G_1). These data suggest that individual PPARγ ligands mediate distinct physiological responses and are indeed SPARM's.

In summary we have made significant progress toward our understanding of the role PPAR plays in breast cancer cells. All three specific aims outlined in the grant proposal have been addressed and reported to the scientific community in the form of manuscripts and national and international presentations. As stated in **aim 1**, we have determined the isoforms of both PPAR and RXR and have found new complexity in the regulation of PPAR that could have implications in the tissue-specific control of expression. Toward understanding the regulation of PPAR expression, **aim 2**, we have demonstrated that there is a significant level of basal PPAR expression in both MCF-7 and MDA-MB-231 cells. Furthermore, the sequence flanking the 5' start site of Exon A1 mediates basal PPAR expression in these cells. We are further defining this region to identify the *cis* regulatory elements responsible for this regulation. In defining the molecular mechanism of transactivation of PPAR, **aim 3**, we have identified a unique form of regulation which involves signal cross talk between PPAR and the estrogen receptor. The mechanism of this unexpected yet potentially important effect and the functional significance are currently under investigation. Our work has also lead us and others [5] to propose the existence of selective PPAR modulators (SPARM's) that will have profound implications on the development of new drugs to combat diabetes and yet not cause an increased risk of breast cancer. This work is ongoing in the lab and state-of-the-art technologies including gene chip analysis are being used to further our understanding of the role PPAR plays in breast cancer. Clearly PPARγ1 is present in breast cancer cells and may provide a direct link between diet and the increased rate of breast cancers seen in this country. These studies may lead to better understanding of the risk of specific dietary components. The data make it clear that fatty acids can indeed function as hormones and this information could lead to important new discoveries impacting dietary guidelines and could be of significant therapeutic value. The implications of these studies could have a profound impact on both prevention and management of this devastating disease.

Methods

Cell Culture: MB-MDA-231, T-47D and MCF-7 were obtained from the ATCC (Rockville, MD) and maintained as described in the attached manuscripts.

Probe Construction: The mouse pSV-Sport/PPARγ2 plasmid was received as a kind gift from Dr. Bruce M. Spiegelman's lab at the Dana Farber Cancer Institute. The hRXRα expression vector (pCMV-RXRα) was a kind gift from Dr. Ron Evans (Howard Hughs Medical Institute, San Diego, CA). Primers were designed to each which aplified a 288 bp and a 321 bp fragment of the Dna binding domain of PPAR and RXR, respectively. The reaction utilized 40 ng of plasmid, 20 pmol of each primer, dNTP (10 mM each) at 0.2 mM, 1.5 mM $MgCl_2$, 1X final concentration polymerase buffer (Promega), and 2.5 units *Taq* Polymerase.

Reporter, Antisense and Mutant ER Plasmid Construction: The reporter construct, PPRE3X-TK-pGL3, contains three copies of a PPRE sequence (AGGACAAAGGTCA) upstream of the minimal thymidine kinase promoter was ligated into the XhoI and HindIII sites of pGL3 basic vector (Promega). This was created by PCR amplification of the region between the 5' XhoI site and the 3' HindIII site of the vector described by Kliewer *et al.* [17]. The amplified product was cloned into the TA cloning vector (Invitrogen, Carlsbad, CA) as per manufacture's instructions and excised with XhoI and HindIII. The *Renilla* control vector (pRL-TK) was purchased from Promega. Antisense expression vector was constructed by amplifying the sequence between $^{+}173$ to $^{+}677$ of the PPAR (L40904) coding sequences. The forward primer was 5' ACCAGTGTTGACACCGAGATCGC 3' and the reverse primer was 5' CTGAAACCGACAGTACTGAC 3'. The amplified product was cloned into the pTarget vector (Promega, Madison, WI). Sequence analysis was used to verify orientation and accuracy of the PCR and subcloning reactions. The human ER expression plasmid lacking the AF2 and AF1 domains, pCMV5 ERα1–530 (ΔAF2) and pCMV4ERαDAB(ΔAF1), respectively, were kindly provided by Dr. Benita Katzenellenbogen. The expression plasmid lacking the DBD, pCMV5ERαAF1-AF2, was constructed by linking two PCR fragments which encode the AF1 and AF2 domains, amino acids 1-174 and amino acids 531–595, respectively. The forward and reverse primers used to amplify the AF1 domain were 5' gaattcggccacggaccatgacc3' (EcoR1) and 5' ggtaccacgttacttcccttgtcattggta3' (Kpn1), respectively. The forwasrd and reverse primers used to amplify the AF2 domain were 5' ggtaccctctatgacctgc3' (Kpn1) and 5' ggatcctcagactgtggcaggg3' (BamH1), respectively. Both were cloned into the TA cloning vector (Promega) and sequenced. The AF2 domain was liberated using KpnI and BamH1 and subcloned into the AF1 plasmid. The entire sequence was then cloned into pCMV5 at the EcoR1 - BamH1 sites.

cDNA Library Construction: Using oligo dT mRNA a cDNA library was created using the Time Saver synthesis kit (Pharmacia) as per instructions. After the second strand synthesis, 5 units of T4 RNA Polymerase were added to the reaction for 1 h at 37°C. The cDNA was then ligated and packaged into the Lambda Zap Express vector (Stratagene) as per instructions. The library was titered at 1.3 x 10^8 plaque forming units/ml (PFU/ml) with ≤15% non-recombinants.

Northern Blot Analysis: Please see attached manuscript, Kilgore *et al.*, MCE.

Transfection and proliferation: Please see attached manuscript, Thoennes *et al.,* In press.

Library Screening: A total of 2x10^5 PFU of phage were incubated at 200 RPM with the bacteria for 15 min at 37°C and plated. Plaques were lifted onto Hybond N+ membranes, denatured, UV crosslinked with 12,000 μJ (Stratagene) and air-dried. Membranes were probed following prehybridization and hybridization steps were performed twice in 2X SSC for 20 min each wash with a final wash in 1X SSC for 20 min. All washes were performed at 65°C. Membranes were then put under Hyperfilm (Amersham) and developed. Dilutions of the phage plugs were then used to plate out for the next round of screening. Third or fourth round of plating was performed to yield

a single population of phage. Helper phage plasmid excision was performed as per instructions that resulted in a pBK-CMV plasmid with cDNA insert.

Sequencing: Plasmid DNA was purified via the alkaline lysis and T4 and T7 universal primers, found in pBK-CMV insert flanking regions, were used during the first round of sequencing,. Sequencing reactions consisted of 400 ng plasmid DNA, 8 µl of ABI Prism Ready Reaction mix (Perkin-Elmer/ABI), 5 pmol of primer, sterile distilled water to a total reaction volume of 20 µl and an oil overlay. Sequencing reactions were run on a ABI 373 Stretch sequencer (Applied Biosystems, Inc.). The primers were based on the published Human PPARγ2 sequence (Accession L40904) in Genbank and on sequences based on our clones.

RACE and RT-PCR: In both cases, single stranded cDNA was synthesized by MMLV reverse transcriptase from polyA$^+$ RNA (Clontech). For the initial reaction an oligonucleotide was used at bases +221 to +243 at the 3' end of exon I. For the RACE library the tailing reaction was performed according to manufactures instructions. In the PCR phase of both reactions the 3' oligonucleotide was between bases +198 to +218 for increased specificity of the PCR reaction. All reactions were carried out following manufactures recommendations (Clonetech). Total RNA was isolated from both MCF-7 and MDA-MB-231 cells. "Ready To Go You Prime First Strand Beads" (Pharmacia Biotech) were used for single-stranded cDNA synthesis, using 20 pmole of the reverse complement primer HPR4 (438-419 of L40904) or HPR4 -10 (428-409) (both are contained within exon II). For the 4 sets of PCR reactions, 20 pmole of each of four 5' primers were used: "5'Greene" (which starts at bp23 of L40904), A1 (starting at bp 95 of L40904), A2 (starting at bp 172) and B (starting at bp 95, [15]). PCR products were ligated into a TA 2.1 cloning vector (Invitrogen) and screened using the BPF1 probe (bp 199-216). Eleven positive clones from the A2 primed population, and eleven clones from the A1 primed population, were identified and sequenced.

RNAse Protection Assays: RNAse protection assays were performed on total cell lysates isolated from control and treated cells as per manufacture's instructions (Ambion, Austin, TX). *In vitro* transcription reaction for RPA probes utilized the MAXIscript kit (Ambion, Austin, TX). One µg of linearized template was used for transcribing antisense RNA probes and T7 RNA polymerase, ^{32}P-UTP, ATP, TTP and CTP were added. The mixture was incubated at 37°C for 30 minutes followed by DNase I digestion for removing the template DNA. The free nucleotides were removed by column purification (Bio-Rad, Hercules, CA). The Molecular Dynamics Fluorescence Scanning System was used to quantitate the bands in the RPA and PPARγ1-specific bands were compared to an 18s rRNA (Ambion, Austin, TX) as loading control.

Cell Sorting / Cell Sorting Experiment Methodology: MCF-7 cells were split and transferred to 12-well plates. After 24 hours following transfer, the media was removed and cells were washed with 1 ml PBS per well. All experiments were started with cells at approximately 50% confluence. One ml of treatment media with or without 20mg/ml 15-deoxy-D12,14-PGJ2 (Cayman Chemical catalog no. 18570) or 10mM Ciglitizone (BIOMOL Research Labs catalog no. GR205) was applied to four wells for every time-point. Upon completion of treatment exposure, all media was removed, and the cells were then subjected to propidium iodide staining for DNA analysis by flow cytometry (reference: Vindelov et al, Cytometry 3., 1983). One ml of solution A trypsinized the cells using 10x Trypsin-EDTA and a stock solution (1g Trisodium citrate dihydrate, 1ml Non-idet P40, 522mg Spermine 4HCL, 60.5mg Tris-hydroxymethyl-aminoethane per liter of distilled water). The cells were then incubated at 37°C for 30 minutes. One ml of solution B containing 10mg trypsin inhibitor and 2mg Ribonuclease A in 20ml of stock solution was added to each well. Again, the cells were incubated for 30 minutes at 37°C. Finally, one ml of solution C (1mg propidium iodide and 23.2mg spermine 4HCL per 20ml) was then added to a 5ml falcon tube containing the suspended cells. Cells were then incubated at 4°C for 60 minutes. All cell cycle phase analyses were performed under the direction of the UK Research Core Facility: Flow Cytometry Laboratory.

Key Research Accomplishments (Entire Grant Period):

- We have demonstrated the several human breast cancer cells express PPARγ1 and RXRα and are functionally responsive to peroxisome proliferators. While we were the first to demonstrate this, and the only group to clone these from breast cancer cells, several other groups have confirmed these findings by other means.
- We have shown that the transcriptional regulation of PPAR expression is under a complex and tissue-specific control. We have cloned one new untranslated first exon and identified another not previously reported in humans. Through subsequent analysis we have demonstrated that the gamma 1 promoter is the predominate driver of basal expression in breast cancer cells.
- We have shown that fatty acids are capable of functioning as ligands for PPAR and can mediate the transcription of target genes.
- We have demonstrated that individual fatty acids are selective modulators of PPAR transactivation that may help explain the difficulty in interpreting the role of diet and breast cancer from the epidemiological data.
- We have demonstrated that PPAR is transcribed, translated and constitutively transactivated in 2 separate lines of breast cancer cells.

Key Research Accomplishments (Final Year of Grant):

- We have now shown that signal crosstalk exists bidirectionally between the ER and PPARγ1. This demonstrates that breast cancer under varying stages of development have the potential to respond to both ER and PPAR ligands is differently..
- We have now solved the genomic structure of PPARγ and placed all know promoters in context. Furthermore, we have clearly demonstrated the source of the majority of PPARγ expression in human breast cancer cells. This will pave the way for additional promoter analysis that is currently being conducted in the lab. Furthermore, these analysis will enable us to construct appropriate vectors to create transgenic animals to help examine the timing and sites of expression that we may better understand the role PPARg plays in normal mammary development and in tumor progression.
- We have shown that several ligands selectively activate PPARγ transactivation. This has lead us to propose the concept now well entrenched the estrogen receptor world of selective PPAR modulators or SPARM's. This is currently being tested further through the use of "Gene Chip" analysis within our core facility at the University of Kentucky. Data from the first set of chips are being mined and support or hypothesis and will lead our work in unforeseen directions. These analyses should facilitate the development of selective drugs useful in the treatment of breast cancer.
- Our work was recognized by an invitation to speak at the Gordon Conference on Hormonal Carcinogenesis in July,2001.

Reportable Outcomes

Training and employment:

1. 4 MS degrees awarded and supported by this grant. All have remained in science or returned to duties with the US Army.
2. 1 postdoctoral fellow recruited into the lab and into breast cancer research.
3. Based upon the work accomplished as a consequence of this grant I was recruited to the University of Kentucky School of Medicine in the Department of Molecular and Biomedical Pharmacology.

Manuscripts:

MW Kilgore, PL Tate, S Rai, E Sengoku and TM Price 1997 MCF-7 and T47D Human Breast Cancer Cells Contain a Functional Peroxisomal Response. Molecular and Cellular Endocrinology, 129:229-235.

SR Theonnes, PL Tate, TM Price and **MW Kilgore** 2000 Differential Transcriptional activation of peroxisome proliferator-activated receptor gamma by omega-3 and omega-6 fatty acids in MCF-7 cells.. Mol Cell Endocrinol. 2000 Feb 25;160(1-2):67-73

Jones GB, Hynd G, Wright JM, Purohit A, Plourde GW 2nd, Huber RS, Mathews JE, Li A, **Kilgore MW**, Bubley GJ, Yancisin M, Brown MA, 2001 Target-directed enediynes: designed estramycins. Journal of Organic Chemistry 2001 Jun 1;66(11):3688-95.

PL Tate, JM Nani TM Price and **MW Kilgore** 2002 Ginseng activates transcription of estrogen responsive genes through the alpha and beta forms of the estrogen receptor. (Submitted, Journal of Endocrinology).

X Wang and **MW Kilgore** 2002 Signal cross-talk between the alpha and beta forms of the estrogen receptor and the peroxisome proliferator-activated receptor in human breast cancer cells. (Submitted, Molecular and Cellular Endocrinology).

MW Kilgore and X Wang 2002 Promoter analysis of PPARg1 expression in human breast cancer cells. (in preparation).

Abstracts presented:

MW Kilgore, PL Tate, S Rai, E Sengoku and TM Price. 1997 MCF-7 and T47D Human Breast Cancer Cells Contain a Functional Response To Peroxisomal Proliferators. (Sponsor, DJ Fernandes) (88th annual meeting of the American Association of Cancer Research, San Diego, CA, April, 1997).

E. Sengoku and **MW Kilgore.** cDNA Cloning of Human Peroxisome Proliferator Activated Receptor from MCF7 Human Breast Cancer Cells. (79th Annual meeting of the Endocrine Society, Minneapolis, MN, June 1997).

Abstracts presented continued:

PL Tate, TM Price and **MW Kilgore.** The Effects of Estrogen on DNA Binding and Transcription Activation of Peroxisome Proliferator Activated Receptor in Human Breast Cancer Cells. (79th Annual meeting of the Endocrine Society, Minneapolis, MN, June 1997).

S Rai, PL Tate and **MW Kilgore.** Dietary Fats Stimulate Peroxisome Proliferator Activated Receptor-Mediated Transcription in MCF7 Human Breast Cancer Cells. (79th Annual meeting of the Endocrine Society, Minneapolis, MN, June 1997).

E Sengoku and **MW Kilgore**. MCF-7 cells express a unique form of the peroxisome proliferator-activated. . Seventh annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC, January, 1998.

S Rai and **MW Kilgore.** Selective activation of the peroxisome proliferator-activated receptor by dietary fatty acids in human breast cancer cells. Seventh annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC, January, 1998.

PL Tate, JM Marchiori and **MW Kilgore.** *Panax ginseng* extract activates the estrogen receptor in breast cancer. (80th Annual meeting of the Endocrine Society, New Orleans, LA, June 1998).

S Rai, PL Tate, TM Price, **MW Kilgore**. Role of dietary fats in PPAR-mediated carcinoma in MCF-7 human breast cancer cell lines. (80th Annual meeting of the Endocrine Society, New Orleans, LA, June 1998).

X Wang, PL Tate, TM Price and **MW Kilgore**. Signal cross talk between the estrogen receptor and the peroxisome proliferator-activated receptor in human breast cancer cells. Eighth annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC, January, 1999.

PT Tate, JM Nani, TM Price and **MW Kilgore**. The response of breast cancer cells treated with ginseng extract. . (81st Annual meeting of the Endocrine Society, San Diego, CA, June 1999).

X Wang, PL Tate, TM Price and **MW Kilgore**. Signal cross talk between the estrogen receptor and the peroxisome proliferator-activated receptor in human breast cancer cells. (81st Annual meeting of the Endocrine Society, San Diego, CA, June 1999).

S Peirce and **MW Kilgore.** A Dominant Negative Mutant of PPARγ Reduces PPRE-mediated Reporter Activity in MCF-7 Human Breast Cancer Cells. Ninth annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC. January 2000.

ER Loghin and **MW Kilgore.** Regulation of Peroxisome Proliferator-Activated Receptor GAMMA (PPARγ) Gene Expression in MCF-7 and MDA-MB-231 Human Breast Cancer Cells. Ninth annual South Carolina Research Conference, Wild Dunes Isle of Palms, SC. January 2000.

X Wang, PL Tate, SR Thoennes, E Loghin, TM Price and **MW Kilgore.** Signal Cross Talk between Estrogen Receptor Alpha and Beta and the Peroxisome Proliferator-Activated Receptor gamma1 in MDA-MB-231 Breast Cancer Cells. Era of Hope, Department of Defense Breast Cancer Research Program Meeting. Atlanta, GA, June 2000.

Abstracts presented continued:

MW Kilgore, X Wang, SR, Thoennes, E Loghin and TM Price. Estrogen Receptor Alpha and Beta Mediate Transcriptional Activation of the Peroxisome Proliferator-Activated Receptor Gamma1 in MDA-MB-231 Breast Cancer Cells. (82nd Annual meeting of the Endocrine Society, Toronto, Canada, June 2000).

X Wang and **MW Kilgore.** Peroxisome proliferator-activated receptor gamma: Novel promoters and transcriptional regulation in human breast cancer cells. . (83rd Annual meeting of the Endocrine Society, Denver, CO, June 2001).

MW Kilgore. Signal crosstalk between the estrogen receptor and the peroxisome proliferator-activated receptor gamma 1 in human breast cancer cells. The Gordon Conference. Kimball Union Academy, NH. July, 2001.

Invited presentations:

Effects of Peroxisome Proliferators in the Human Breast: A Molecular Model for the Role of Diet in Breast Cancer. Carolinas Medical Center. Charlotte, NC, March 1998.

Peroxisome proliferator-activated receptor putative link between the environment and cancer. Medical University of South Carolina, August, 1998

Effects of peroxisome proliferators on human breast cancer cells: A molecular model to examine the link between diet and breast cancer. University of Texas Health Center at Tyler. January 1999.

Effects of peroxisome proliferators on human breast cancer cells: A molecular model to examine the link between diet and breast cancer. Clemson University Institute for Environmental Toxicology. March 1999.

Molecular mechanisms of nuclear receptor transactivation in human breast cancers cells by dietary fatty acids and phytoestrogens. University of Louisville School of Medicine. April, 1999.

Molecular mechanisms of peroxisome proliferator-activate receptor transactivation in MCF-7 and MDA-MB-231 human breast cancer cells. Texas Tech University HSC. August, 1999.

Molecular mechanisms of nuclear receptor in human breast cancer cells by dietary acids and phytoestrogens. MD Anderson Cancer Center, Research Park, Department of Molecular Carcinogenesis. November, 1999.

The Role in the peroxisome proliferator-activated receptor in the human mammary gland. University of Colorado Health Sciences Center. June 2001.

Signal crosstalk between the estrogen receptor and the peroxisome proliferator-activated receptor gamma 1 in human breast cancer cells. The Gordon Conference. July, 2001.

Description of Training:

To date four students have received their masters' degrees (MS) as a result of this award. Ms Sudha Thoennes is currently a technician at the University of Florida, Gainesville Medical School and is investigating viral mechanisms of oncogenesis. Mr. Eiichi Sengoku is currently Product Manager for North American Operations at Nalgen in Rochester New York. Major Mark Corbett is serving in the US Army in the Chemical Corp following completion of his degree. Ms. Evelina Loghin is a technician on a DARPA funded project at Clemson University's Department of Bioengineering. Ms. Rupalika Singh, an undergraduate student, has worked in the lab for the last 2 semesters has been accepted to medical school in part based upon her research experience gained in the my lab as a consequence of this grant support. Dr. Xin Wang has been in the lab as a postdoctoral fellow since October 1998 and has already presented two abstracts at international conferences. She continues to progress well and is currently writing a manuscript which will be submitted this spring. Also as a consequence of this grant and the data generated from its funding I was recruited to the University of Kentucky School of Medicine in the department of Molecular and Biomedical Pharmacology. This is a much better research facility with core facilities and collaborative faculty. I have also been accepted to the faculty of the Markey Cancer Center. I was also pleased to have Dr. Wang join me in this move and she continues to progress well.

Conclusions

These data indicate that human breast cancer cells express PPARγ and respond to PPAR ligands by upregulating the expression of target genes. The implication of these findings is particularly important in light of the wide spread use of thiazolidinediones (TZDs) in the treatment of type-2 diabetes, which are themselves ligands for PPARγ. Furthermore, we have demonstrated that dietary fatty acids shown to bind PPARγ can regulate transactivation in these cells. This suggests that fatty acids can function as hormones and mediate transcription of target genes in the breast. The fact that we observe selective activation by different classes of fatty acids may indicate that they have differential functions in the breast. Whether these or other ligands play a role in breast cancer development or prevention must now be addressed.

Our demonstration that ER and PPARγ signal transduction pathways interact could have profound implications in the treatment and prevention of breast cancer. Clearly, the ER status of a breast tumor is used as the principle indicator for treatment with Tamoxifen and other antiestrogen. What is not clear is whether all the therapeutic benefits from these drugs are direct effects through the ER or whether they are mediated through other receptor systems. The fact that ER downregulates the ability of PPARγ to mediate gene transcription could have important physiological consequences and these must be examined. Furthermore, the role PPARγ plays in normal development of the mammary gland and in the development or progression of breast cancer must too be examined. These studies could provide an important new target in our battle against breast cancer.

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Signal cross-talk between estrogen receptor alpha and beta and the peroxisome proliferator-activated receptor gamma1 in MDA-MB-231 and MCF-7 breast cancer cells.

Running title: Crosstalk between PPARγ1 and ERα and β.

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Abstract

We have previously demonstrated that PPARγ is expressed and transcriptionally responsive to both synthetic and natural ligands in a variety of human breast cancer cells. We also observed that the ability of ligands to transactivate PPAR correlates with the expression of the estrogen receptor. While previous reports indicate that PPARγ can mediate the expression of estrogen target genes, no data has suggested that ER expression can alter the transcriptional regulation of PPARγ target gene expression. Here we have demonstrate that the expression of either ERα or ERβ significantly inhibits transactivation of PPAR by thiazolidinediones and also lowers basal reporter activity. Interestingly, the presence of an ER antagonist does not inhibit this response while estradiol treatment inhibits the ligand-stimulated transactivation of PPARγ in cells expressing ERα but not ERβ. Cells transfected with ERα deletion mutants demonstrate that the DNA binding domain of the ER is required to repress PPAR transactivation in these cells. Finally, using RNase protection assays we show that the inhibition of PPAR function is not due to a decrease in the expression of PPARγ. These data suggest that signal cross talk exists bidirectionally between PPARγ and ER in breast cancer cells.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are a family of transcription factors that belong to the nuclear receptor superfamily [Mangelsdorf, 1995 #151]. Transactivation of these receptors follows ligand binding, nuclear localization and heterodimerization with the 9-*cis* retinoic acid receptor (RXR) [Tontonoz, 1994 #64; Issemann, 1993 #213]. PPAR has been cloned from Xenopus to man [Schmidt, 1992 #197; Sher, 1993 #194; Tontonoz, 1994 #65; Mukherjee, 1994 #162; Mukherjee, 1997 #72; Elbrecht, 1996 #75; Chen, 1993 #163]. Three distinct isoforms, termed α, β, (also called δ, NUC-1 and FAAR) and γ have been cloned (NR1C1, NR1C2 and NR1C3, respectively) [, 1999 #341]. Each is encoded by separate genes and show a distinct tissue distribution of expression and ligand binding specificity [Kliewer, 1994 #195; Mukherjee, 1997 #72; Auboeuf, 1997 #160; Braissant, 1996 #166]. PPARα is expressed in liver, kidney, heart and brown adipose tissue [Braissant, 1996 #166] and mediates the regulation of genes involved in oxidation of fatty acids and detoxification of several xenobiotics [Latruffe, 1997 #398]. PPARβ is expressed in a wide range of tissues and is thought to play a role in lipid metabolism, regulation of high density lipoprotein (HDL) levels [Gelman, 1999 #153] and mediate oligodendrocyte maturation and myelin sheath formation [Granneman, 1998 #205]. PPARγ is prodominately expressed in adipoctyes where it mediates differentiation [Tontonoz, 1994 #65]. PPARγ is thought to be the primary target of thiazolidinediones in the regulation of insulin sensitivity [Lehmann, 1995 #203; Berger, 1996 #202]. Animal studies also indicate that PPARγ plays a pivotal role in placental function [Barak, 1999 #325] and uptake of oxidized low-density

lipoproteins (LDLs) [Marx, 1998 #167; Nagy, 1998 #133; Tontonoz, 1998 #157; Xin, 1999 #140]. In human breast cancer cells PPARγ is expressed at high levels and is functionally responsive to synthetic and natural ligands [Kilgore, 1997 #1; Thoennes, 2000 #235]. PPARγ expression has also been reported in both primary and metastatic breast cancer where transactivation has been reported to induce differentiation [Mueller, 1998 #4] and apoptosis [Elstner, 1998 #3].

Human PPARγ, originally cloned form hematopoietic stem cells [Greene, 1995 #77], is under complex and tissue-specific regulation. Promoters elements are thought to be associated with at least 4 unique first exons, three of which are untranslated [Fajas, 1997 #71; Fajas, 1998 #113; Greene, 1995 #77, unpublished observations]. At the protein level, PPARγ has two isoforms termed γ1 and γ2 that arise as a function of differential promoter usage from a common gene [Zhu, 1995 #199; Fajas, 1997 #71]. Gamma 2 differs from γ1 by the use of an in-frame ATG start site of translation, located in an exon unique to γ2, resulting in an additional 28 N-terminal amino acids [Mukherjee, 1997 #72]. The functional significance of these amino acids remains unknown. In each case, PPAR heterodimerizes with the retinoid X receptor alpha (RXRα) [Keller, 1995 #122] which, in turn, is the receptor for 9-*cis* retinoic acid [Mangelsdorf, 1990 #164]. Originally cloned and classified as an orphan receptor, the endogenous ligand(s) for PPARγ is still unclear. Compounds that can bind and mediate transcriptional activation include prostaglandin J_2 [Forman, 1995 #96; Kliewer, 1995 #97], thiazolidinediones [Berger, 1996 #202; Fajas, 1998 #113; Lehmann, 1995 #203] and fatty acids [Gottlicher, 1992 #215; Issemann, 1993 #213; Keller, 1993 #214, Kliewer, 1997 #387; Thoennes, 2000 #235].

Recently it has been reported that PPARγ is capable of interacting with the estrogen receptor (ER) signal transduction pathway. PPAR binds DNA as a heterodimer with the retioid X receptor alpha (RXRα) where it binds response elements (PPRE) in target genes. Although homodimeric binding has recently been reported, this has been demonstrated *in vitro* only [Okuno, 2001 #391]. PPREs consist of a direct tandem repeat core element with a single intervening base [Mangelsdorf, 1995 #151]. Wahli and co-workers demonstrated that the estrogen response element, which drives the expression of vitellogenin A2 gene, can also function as a PPRE [Keller, 1995 #122]. Although the PPAR/RXR complex was shown to bind several other ERE-related palindromic response elements, only the vitellogenin ERE is a functional PPRE in transcriptional reporter assays [Keller, 1995 #122]. Non-permissive binding of PPAR/RXR inhibits transactivation by the ER through competitive binding [Keller, 1995 #122]. Binding to an ERE was also demonstrated in human reproductive tissues and shown to be independent of the estrogen receptor [Nunez, 1997 #103]. By contrast, little is known regarding the ability of the ER to interact with the signal transduction pathway of PPAR.

In our investigation of PPAR transactivation in both MCF-7 cells and MDA-MB-231 cells, we observed significant differences in their responsiveness to PPAR ligands. Since MCF-7 cells inherently express high levels of ERα and MDA-MB-231 express very low, if any, ERα or ERβ, we sought to determine if the presence of ER altered transactivation of PPARγ. Here we report that the expression of either ERα or ERβ subtype is capable of inhibiting PPAR-mediated activation in human breast cancer cells. The ER requires a functional DNA binding domain to exhibit this effect but does not appear to be strictly dependent on agonist or antagonists of ER. As PPAR agonist are

currently being developed for therapeutic intervention in breast cancer, these findings could be of critical importance in effective drug design.

Materials and Methods

Materials:

MCF-7 and MDA-MB-231 breast cancer cells were obtained from American Type Culture Collection (Rockville, MD). Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and ^{32}P dCTP (1.0 mCi/mol) was purchased from NEN (Boston, MA). 17β-Estradiol (E_2) and all other standard chemicals were obtained from Sigma (St. Louis, MO). The pure anti-estrogen ICI 182,780 (ICI) was generously provided by Zeneca Pharmaceuticals (Cheshire, England). The developmental thiazolidinedione BRL 48,482 (BRL), a selective PPARγ agonist, was a kind gift from GlaxoSmithKline (Research Triangle Park, NC). The human ERα plasmid (pRST7-ERα) was a kind gift from Dr. Geoffrey Greene of the Ben May Institute, Northwestern University, and the human ERβ expression vector (pCMV5-hERβ) was generously provided by Dr. J.Å. Gustafsson, Karolinska Institute, Sweden. The aryl hydrocarbon receptor (hAhR) was a gift from Dr. Hollie Swanson, University of Kentucky College of Medicine and the androgen receptor (HAR) was generously provided by Dr. Albert Brinkmann, Erasmus University, Rotterdam, The Netherlands.

Cell Culture:

MDA-MB-231 cells were routinely cultured in IMEM (Biofluids, Rockville, MD) containing 10% TCH serum replacement medium (Celox Laboratories, Inc., St. Paul, MN) and MCF-7 cells were cultured in DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 2% TCH and 0.5% fetal bovine serum (FBS, Hyclone). Both cell types were grown in medium lacking phenol red at 37°C in a 5% CO_2 atmosphere. Cells were grown in T-75 flask before transferred to 12-well plates (Corning) in preparation for transfection.

Reporter, Antisense and Mutant ER Plasmid Construction:

The reporter construct, PPRE3X-TK-pGL3, contains three copies of a PPRE sequence (AGGACAAAGGTCA) upstream of the minimal thymidine kinase promoter was ligated into the XhoI and HindIII sites of pGL3 basic vector (Promega). This was created by PCR amplification of the region between the 5' XhoI site and the 3' HindIII site of the vector described by Kliewer *et al.* [Kliewer, 1992 #53]. The amplified product was cloned into the TA cloning vector (Invitrogen, Carlsbad, CA) as per manufacture's instructions and excised with XhoI and HindIII. The *Renilla* control vector (pRL-TK) was purchased from Promega. Antisense expression vector was constructed by amplifying the sequence between $^{+}173$ to $^{+}677$ of the PPAR (L40904) coding sequences. The forward primer was 5' ACCAGTGTTGACACCGAGATCGC 3' and the reverse primer was 5' CTGAAACCGACAGTACTGAC 3'. The amplified product was cloned into the pTarget vector (Promega, Madison, WI). Sequence analysis was used to verify

orientation and accuracy of the PCR and subcloning reactions. The human ER expression plasmid lacking the AF2 and AF1 domains, pCMV5 ERα1–530 (ΔAF2) and pCMV4ERαDAB(ΔAF1), respectively, were kindly provided by Dr. Benita Katzenellenbogen. The expression plasmid lacking the DBD, pCMV5ERαAF1-AF2, was constructed by linking two PCR fragments which encode the AF1 and AF2 domains, amino acids 1-174 and amino acids 531–595, respectively. The forward and reverse primers used to amplify the AF1 domain were 5' gaattcggccacggaccatgacc3' (EcoR1) and 5' ggtaccacgttacttcccttgtcattggta3' (Kpn1), respectively. The forwasrd and reverse primers used to amplify the AF2 domain were 5' ggtaccctctatgacctgc3' (Kpn1) and 5' ggatcctcagactgtggcaggg3' (BamH1), respectively. Both were cloned into the TA cloning vector (Promega) and sequenced. The AF2 domain was liberated using KpnI and BamH1 and subcloned into the AF1 plasmid. The entire sequence was then cloned into pCMV5 at the EcoR1 - BamH1 sites.

Transient Transfection Analysis:

MCF-7 cells were transiently transfected with 5μg of a 3xPPRE-TK-pGL3 reporter vector and 0.5 μg pRL-TK (Promega) per plate by the $CaPO_4$ DNA precipitation. MDA-MB 231 cells were transfected with 5 μg of reporter plasmid and 0.5 μg control plasmid DNA using Lipofectin (GIBCO). In ER expression experiments, cells were cotransfected with 6 μg of an expression vector coding for of wild type ERα, ERβ expression vectors or pBluescript™ to maintain equal amounts of DNA in each transfection. Cells were subsequently treated with or without 17β-estradiol (E_2, 1 nM), BRL (50 μM) and ICI (10

μM) alone or in combination for 18-24 hours. Following treatment, cells were lysed in 100 μl passive lysis buffer and treated according to manufacturer's instructions (Promega, Analytical Luminescence Laboratory). Luminometry was performed on a Monolight 2010 and data calculated as raw luciferase units divided by raw *Renilla* units (RLU's). Mean fold induction was obtained by dividing the RLU data from each treatment well by the mean control values. For statistical analysis each set of treatments was performed in replicates of three in three separate experiments and analyzed by ANOVA for random block design and statistical significance assessed by Tukey's analysis. Differences were set at $p \leq 0.05$ confidence limits.

Electrophoretic Mobility Shift Assay:

Nuclear extracts from untransfected MDA-MB-231 cells and 231 cells transfected with either ERα or ERβ were isolated and run on non-denaturing gel using a radiolabeled PPRE as target. A double stranded PPRE was prepared by annealing the sense and antisense-oligonucleotides: 5' GGGACCAGGACAAAGGTCACGTT 3' and 5'GGGAACGTGACCTTTGTCCTGGTC 3', respectively. Probes were created by extending the nucleotides with klenow enzyme in the presence of [α^{32}P] dCTP (3000 Ci/ mmol; New England Nuclear, Boston, MA). As a control for non-specific binding, a cold PPRE competitor was included. Gel mobility shift analysis were performed with 10 μg of nuclear extract incubated in a final volume of 20μl containing 1mg/ml poly dI-dC, 0.5 mM PMSF, 24 mM Leupetin, 0.15mM Pepstatin, 0.3mM Aprotonin (Pharmacia, Piscataway, NJ), 75 mM KCl, 20 mM HEPES (pH7.9), 0.2 mM EDTA (pH8.0) and 20%

glycerol. Samples were incubated on ice for 15 minutes. Ten thousand CPMs of labeled oligonucleotide with or without 32-fold molar excess of cold competitor were added and incubated for an additional 15 minutes at room temperature. The reaction mixture was loaded directly onto a 1.5 mm thick, 4% polyacrylamide gel (37.5:1, acrylamide:bisacrylamide) in 0.5x TBE buffer and run at 120 V. Following eletrophoresis, gels were fixed in 10% Methanol and 10% Acetic Acid for 30 minutes, dried on a Watman filter paper and exposed to BioMax MR (Eastman Kodak, Rochester, NY) film at -80°C with an intensifying screen.

RNase Protection Assay (RPA):

RNAse protection assays were performed on total cell lysates isolated from control and treated cells as per manufacture's instructions (Ambion, Austin, TX). *In vitro* transcription reaction for RPA probes utilized the MAXIscript kit (Ambion, Austin, TX). One μg of linearized template was used for transcribing antisense RNA probes and T7 RNA polymerase, ^{32}P-UTP, ATP, TTP and CTP were added. The mixture was incubated at 37°C for 30 minutes followed by DNase I digestion for removing the template DNA. The free nucleotides were removed by column purification (Bio-Rad, Hercules, CA). The Molecular Dynamics Fluorescence Scanning System was used to quantitate the bands in the RPA and PPARγ1-specific bands were compared to an 18s rRNA (Ambion, Austin, TX) as loading control.

Results

Both MCF-7 and MDA-MB-231 cells were transiently transfected with a luciferase reporter under the control of three copies of a PPRE using the TK minimal promoter. As a control for cell numbers and transfection efficiency, cells were co-transfected with the *Renilla* vector under constitutive regulation of the HSV-TK promoter. The thiazolidinedione BRL 48,482 significantly stimulated reporter activity in both cells although the basal and stimulated levels of reporter activity are significantly higher in the 231 cells than in MCF-7 cells. To examine the specificity of the three PPRE's in mediating these responses, each cell type was transfected with a null vector containing the entire luciferase reporter plasmid with the minimal TK promoter but lacking the PPREs. In each case no stimulation was seen with the null reporter construct (data not shown). To determine whether the PPRE reporter is mediated by PPARγ, an antisense (AS) expression vector was constructed to reduce the endogenous levels of protein expression. The antisense expression vector is specific for all forms of PPARγ, spanning bases +173 to +685 (L40904). Although the AS would block the translation of PPARγ1, γ2 and γ3 [Fajas, 1998 #113] we have been able to detect only γ1 expression in these cells by several methods (data not shown). In each case, expression of the antisense completely blocked the BRL stimulated increase in reporter activity. Furthermore, expression of the antisense inhibited reporter activity to levels below that of control in non-stimulated cells in both cell types.

Since MCF-7 and MDA-MB-231 cells differ in their responsiveness to individual PPAR ligands, we sought to examine the molecular basis of this effect. One important difference between these cells is their ER status. While MDA-MB-231 express very low levels of ER, if any, MCF-7 cells express high levels of ERα [Lazennec, 1999 #397]. To determine whether the expression and transactivation of the ER alter the transcriptional activity of PPAR, we co-transfected either ERα or ERβ expression vectors into MDA-MB-231 cells along with the PPRE-mediated transcriptional reporter and the *Renilla* control. Cells not transfected with the ER plasmids received pBluescript DNA such that all cells were transfected with equal amounts of DNA. Expression of either ERα or ERβ significantly inhibited basal reporter activity (figures 2 and 3). Interestingly, ERβ inhibited reporter activity in unstimulated cells significantly greater than ERα. In cells treated with BRL (figures 2 and 3), PPRE-mediated transcriptional reporter activity was significantly greater than control but expression of either ER subtype blunted this response. To determine if either form of the ER required hormone to mediate reporter activity, cells were treated with BRL, E_2 or a combination of both (figure 2). Co-treatment had no effect on cells lacking ER or expressing ERβ, however, in cells expressing ERα, E_2 plus BRL treatment significantly inhibited reporter activity below BRL alone. No differences were seen in cells expressing either from the ER when treated with E_2 compared to control. In a similar set of experiments we sought to determine whether a "pure antagonist" of ER action could alter PPAR transcriptional activity (figure 3). As was seen in figure 2, the presence of either form of the ER significantly inhibited reporter and again ERβ is a more potent inhibitor of transcriptional activation than ERα. ICI treatment did not alter the inhibitory effects of either ERα or ERβ on reporter activity

compared to untreated cells. In cells treated with both ICI and BRL no differences were seen in those expressing ERα or ERβ compared to BRL treatment alone. By contrast, however, treatment of cells with BRL and ICI significantly inhibited reporter activity compared to cells not expressing ER stimulated with BRL alone.

To determine whether the expression of ERα or β was capable of rendering these cells functionally responsive to estrogen treatment, cells were transfected with an estrogen response element (ERE) driving the luciferase reporter and with each ER expression vector (figure 4). Cells were treated with or without (C) 17β-estradiol (E_2) and ICI. Whereas 231 cells were unresponsive to estrogen treatment, expression of either form of the ER resulted in the upregulation of reporter gene activity following 17β-estradiol treatment. Addition of the anti-estrogen ICI completely inhibited reporter gene activity.

To address the molecular mechanism whereby ER inhibits PPARγ transactivation, we have examined DNA binding (figure 5) to a PPRE. Nuclear proteins were isolated from MDA-MB-231 from cells transfected with or without an ERα or ERβ expression plasmid. Using a radiolabeled PPRE, gel shift analysis was performed with each of the three nuclear extracts. To assess the specificity of binding, a 32 fold molar excess of cold PPRE was used as competitor (+ lanes). As seen in figure 5 (- lanes), the presence of either ERα or ERβ did not inhibit DNA binding suggesting that the ER does not prevent PPAR from recognizing and binding to its response element. Northern blot analysis, like that of transient transfection analysis, indicates that of both forms of the ER are being expressed (data not shown). Furthermore, nuclear extracts from cells transfected with either ERα or β expression plasmids specifically bound and altered the mobility of a

labeled ERE. By contrast, nuclear extracts from cells transfected with the pBluescript plasmid did not exhibit ERE binding (data not shown).

We further sought to assess whether inhibition of PPARγ transactivation is ER-specific or whether the decrease in PPAR transactivation is due to non-specific squelching. We chose a closely related nuclear receptor, the human androgen receptor (AR) and a basic helix-loop-helix transcription factor the aryl hydrocarbon receptor (hAhR). MCF-7 cells were transfected with or without (C) hAR or hAhR expression plasmids. Unlike ER expression, transfection of cells with either nuclear receptor did not inhibit basal PPRE-mediated reporter activity as was seen with both forms of the ER (figure 6).

To further define the mechanism of PPAR inactivation we have examined the domains of the ER involved in repression of reporter activity. Mutant forms of the ERα have been created lacking the AF1 domain, the AF2 domain or the DNA binding domain (DBD). In each case the ability of wild type and ER mutants to mediate both basal and BRL-stimulated transactivation of PPARγ has been assessed (figure 7). As seen previously, the wild type ERα (row 2) containing all three domains significantly inhibited both basal and BRL-stimulated PPAR transactivation. Deletion of the AF1 domain (row 3) or AF2 domain (row 4) did not alter their ability to inhibit basal and stimulated reporter activation. The deletion of the DBD domain (row 5), however, rendered this deletional mutant unable to inhibit either basal or stimulated PPAR transactivation. To examine both the expression and the function of this DBD mutation we have transfected this mutant and the wildtype ERα into MDA-MB-231 cells and measured estrogen responsiveness (figure 8). As seen in figure 4, when transfected with

an ERE-mediated reporter plasmid, expression of the wildtype ERα imparts estrogen responsiveness to these cells. The DBD mutant, by contrast, is unresponsive to transactivation by estrogen treatment. When co-expressed, the DBD mutant is able to attenuate the reporter activation by the wildtype ERα.

Since the inhibition of PPAR responsiveness in cells expressing the ER could be due to ER mediated down regulation of PPAR expression, we have examined this possibility. First, using RNase protection assays, we have shown that the only significant expression of PPARγ is that of the promoter associated with γ1 (data not shown). Therefore, we have used this probe to quantitate PPARγ mRNA and examine the regulation of expression in MCF-7 cells following estrogen treatment. These experiments were conducted in the presence and absence of cycloheximide to determine whether indirect regulation of PPARγ1 is mediated by estrogen treatment in these cells. Figure 9 clearly demonstrates that treatment of these cells with physiological concentrations of 17β-estradiol did not result in the down regulation of PPAR expression with or without new protein synthesis. These data suggest that the ER directly inhibits transactivation or DNA binding and thus target gene transcription by PPARγ.

Discussion

Initially, the primary role of PPARγ was thought to be in mediating the differentiation of adipose stormal cells into adipocytes [Tontonoz, 1994 #65]. As this was thought to be a universal mechanism of PPARγ ligands our demonstration of a functional PPAR response in human breast cancer cells [Kilgore, 1997 #1] generated a

great deal of interests in using PPARγ-specific ligands for the treatment of breast cancer. Various ligands have been reported to induce apoptosis [Elstner, 1998 #3; Clay, 1999 #226], initiate cell cycle withdrawal [Elstner, 1998 #3; Yin, 2001 #449] and mediate the transcription of target genes consistent with a more differentiated, less malignant phenotype [Mueller, 1998 #4]. We, however, have observed that some ligands which bind and transactivate PPARγ do not always correlate with a decrease in proliferation [Thoennes, 2000 #235]. This has lead us and others to speculate that like selective estrogen receptor modulators or SERM's PPARγ ligands may also selectively regulate receptor function and thus cellular responses [Sporn, 2001 #445]. On the other hand, differences in cellular responses to individual ligands between tissues can be atributed to differences in the expression of co-activators, co-repressors and other nuclear receptors that can contribute to, or inhibit transactivation [Katzenellenbogen, 2000 #382; Oakley, 1999 #458; Shi, 2001 #460]. We too have observed that the activation of a transcriptional reporter by individual PPARγ ligands is significantly different between human breast cancer cell lines. This raises the possibility that the expression of proteins capable in interacting with the PPARγ signaling pathway differ between these cells and these differences may alter PPARγ transactivation. Therefore, we reasoned that examination of the functional consequences of these differences might shed light on the molecular mechanisms of PPARγ transactivation.

Many human breast cancers present as ER positive only to become unresponsive to estrogen or to exhibit estrogen independent growth. The latter conditions are of particular interest since these are more difficult to treat using standard endocrine therapies. MCF-7 cells and MDA-MB-231 are used as models of these conditions as

they are ER positive and negative, respectively. Although MDA-MB-231 cells do not express ERα some controversy remains whether they express ERβ. Several reports present evidence for low levels of expression but we have been unable to observe a response to 17β-estradiol using a consensus estrogen response element (ERE) driving the luciferase reporter describe here. Co-transfection with an ERα or β expression vector, however, restores the ERE-mediated responsiveness indicating the lesion is due to ER expression itself and not other co-activators. If MDA-MB-231 cells express either form of ER, we believe it is too low to detect using the luciferase reporter. Furthermore, when using a radiolabeled ERE no band is detected in gel shift analysis unless cells were first transfected with an ER expression vector prior to isolation of nuclear proteins.

The studies described in this manuscript highlight several important aspects of PPARγ function in breast cancer cells. First, these data indicate that PPARγ is transcribed and transactived at some basal level in both MCF-7 and MDA-MB-231 cells. Previously we reported that ω-3 fatty acids, which bind PPARγ [Kliewer, 1997 #387], inhibited reporter activity to levels below that of control [Thoennes, 2000 #235]. While these fatty acids may result in receptor inactivation or they may compete with some endogenous ligand for receptor binding, we have no direct support of this hypothesis. The fact that the antisense expression vector alone was able to reduce reporter activity below control levels, however, provides direct support for the hypothesis that the receptor is continuously transcribed and translated at some basal level otherwise only stimulated reporter activity would have been affected. This also suggests that human breast cancer cells synthesize an endogenous ligand for PPARγ, RXRα or both. These experiments also validate the specificity of the PPRE reporter as a faithful indicator of PPARγ

transactivation. Furthermore, we have also shown that both cell lines are functionally responsive to several PPARγ ligands. When the data is compared between cells, however, it is clear that MCF-7 cells have lower basal and stimulated reporter activity. The fact that there is data indicating that PPAR is able to bind estrogen response elements and that ER and PPAR share cofactors necessary for mediating transcription led us to examine whether the ER can interact with the PPARγ signal transduction pathway.

While previous reports indicate that PPARγ can regulate ER target gene expression, these data indicate that signal crosstalk between the ER and PPARγ is bi-directional. The expression of ER α or β in MDA-MB-231 is sufficient to inhibit BRL 48,482-stimulated transactivation of PPARγ. While the expression of ERα is sufficient to significantly repress both basal and stimulated transcriptional regulation of the PPRE-reporter, E_2 treatment further repressed the BRL-stimulated response but not the basal repression. By contrast the repressive ability of ERβ is independent of E_2 treatment. Interestingly, ICI, a pure antagonist of ER action, had no effect on the ability of either ERα or ERβ to mediated reporter activity. The fact that ICI inhibited reporter activity of BRL-stimulated transcription in the absence of ER may suggest that ICI can interact directly with PPARγ. While it is interesting to speculate that ICI directly binds to, and inhibits, PPAR function, this remains to be determined and to our knowledge no binding studies have been preformed. Clearly confirmation of this possibility will require further investigation.

The molecular mechanism of ER suppression of PPAR transactivation remains unclear. While we could not observe direct competition for DNA binding sites the DNA binding domain of ERα is necessary for transcriptional repression. Clearly inhibition

could be mediated through competition for co-activators, increased availability of co-repressors or some other mechanism [Zhu, 2000 #316]. While ER repression of PPARγ's ability to mediated reporter gene expression is not due to down regulating the transcription of PPARγ itself, expression of the ER in these cells could result in the decreased production of an endogenous ligand. This possibility, however, does not seem likely since neither Estradiol nor ICI had a marked impact on reporter activation. The more reasonable explanation may be that the ER is working through a non-classical mechanism. The ER has recently been shown to both positively [Paech, 1997 #463] and negatively [Boffelli, 1999 #218] regulate gene expression by mechanisms not requiring direct ER-DNA binding. The repression we report here could be due to a similar mechanism.

While these data implicate the ER in altering the expression of a reporter element, the range of genes affected is not known. Clearly the nature of the receptor and the DNA binding element are critical in mediating transcriptional regulation. PPAR has been shown to bind some ERE's and upregulate transcription while binding to other ERE's in a non-permissive structure thus preventing transcription [Keller, 1995 #122]. An ERE is an inverted repeat containing 3 intervening bases whereas a PPRE is an imperfect direct repeat with a single intervening sequence [Driscoll, 1998 #464]. The perfect repeat (DR1) also functions as a PPRE in MCF-7 cells [Kilgore, 1997 #1]. Nonetheless, each of these sequences contains an AGGTCA half site, which could be recognized by either an ER or PPARγ. While these ligand-receptor complexes tolerate some variability in the sequence of their response elements, many genes that are positively regulated do not have an apparent consensus response element. Clearly, our understanding of what constitutes

an ERE or a PPRE is under constant refinement and DNA binding should never be considered synonymous with transcriptional regulation. Furthermore, the use of plasmid DNA in assessing gene regulation, while a useful screening tool, should be further characterized by examining the expressions of endogenous genes.

The fact that transactivation PPARγ is mediated by different ligands and by differences in the expression profiles of other receptors like the ER have far reaching implications for drug design and tissue-specific responsiveness. This suggests that these selective PPAR modulators or SPARM's have the potential to uniquely mediate PPARγ both positively and negatively in different tissues. As we continue to understand the actions of thiazolidinediones (TZDs) and other PPARγ ligands on adipocytes, monocytes and, potentially, mammary epithelia, the action in all these tissues must be addressed. In general human breast tumors are ER positive in their early stages of tumorgenesis and only in latter stages of malignant development do a significant number become ER negative. Many of these ER negative tumors have a more aggressive phenotype. The physiological consequences of loss of ER function in advanced stages of many breast tumors make the interactions of ER and PPAR of potential clinical importance. Whether expression and transactivation of PPARγ1 plays an important physiological role in the development or progression of breast cancer awaits further analysis as does significance of the signal cross-talk described here.

Figure Legends

Figure 1. **Transient transfection analysis.** Effects of BRL 48,482 and transfection of a PPARγ antisense expression vector on PPRE-mediated reporter activity in MDA-MB-231 and MCF-7 cells. Cells were plated into 12-well plates and transiently transfected with equal amounts of plasmid DNA with a PPRE-mediated reporter plasmid and a constitutive *Renilla* reporter with or without the antisense expression vector. Cells were then treated with or without 5 μM BRL for 24 hrs and subjected to luminometric analysis. Data from three separate experiments (n=4 per day) was subjected to 2-way ANOVA followed by Tukey's pair-wise comparison and reported as the treatment means ± one-half the critical value for comparison. Bars that share a letter designation are statistically indistinguishable while those that have different letter designations are significantly different ($p \leq 0.05$). Although BRL treatment significantly stimulated reporter activity above control levels in both cell lines, PPRE-mediated reporter activity in MCF-7 cells was significantly lower than in MDA-MB-231 cells. Expression of the PPARγ antisense significantly inhibited both basal and BRL-stimulated reporter activities in both cells.

Figure 2. **Transient transfection analysis.** The effects of ER expression on PPAR-mediated transcriptional activity in MDA-MB-231 cells. Cells were transiently transfected with equal amounts of DNA containing a PPRE-mediated luciferase reporter and the constitutive *Renilla* with or without (C) an ERα (α) or an ERβ (β) expression vector (plasmid). Cells were then treated with or without BRL, 17β-estradiol (E_2), or

both. Data are presented as treatment means from 3 separate experiments ± one half the critical value for comparison ($p \leq 0.05$) as determined by ANOVA followed by Tukey's analysis. Bars that share a letter designation are statistically indistinguishable while those that do not are significantly different. The expression of ERα or β was sufficient to inhibit PPAR transactivation in control, BRL-treated cells and in cells treated with BRL+E_2. Estrogen treatment significantly repressed the BRL stimulated reporter activity in cells expressing ERα but not β. By contrast, E_2 treatment had no effect on reporter activity in cells expressing either form of the ER compared to unstimulated controls.

Figure 3. **Transient transfection analysis.** The effects of ER expression and an ER antagonist on PPAR-mediated transcriptional activity in MDA-MB-231 cells. Cells were transfected (plasmid) with a PPRE-mediated luciferase reporter and the constitutive *Renilla* with or without (C) an ERα (α) or an ERβ (β) expression vector. Each set of cells were then left untreated (Control) or treated with ICI, BRL, or both. Data are presented as treatment means from 3 separate experiments ± one half the critical value for comparison ($p \leq 0.05$) as determined by ANOVA followed by Tukey's analysis. Bars that share a letter designation are statistically indistinguishable while those that do not are significantly different. ICI had not effect on untreated or BRL-stimulated reporter activity in cells expressing ERα or ERβ. By contrast, ICI suppressed PPAR transactivation in BRL stimulation in cells lacking ER expression.

Figure 4. **Transient transfection analysis.** The effects of ERα or ERβ expression on estrogen and antagonist treatment in MDA-MB-231 cells. . Cells were

transfected (plasmid) with a ERE-mediated luciferase reporter and the constitutive *Renilla* with or without (Control) an ERα or an ERβ expression vector. Each set of cells were then left untreated (Control) or treated with ICI, 17β estradiol (E_2), or both. Data are presented as treatment means from 3 separate experiments ± one half the critical value for comparison ($p \leq 0.05$) as determined by ANOVA followed by Tukey's analysis. Transfection of cells with either ERα or ERβ re-established estrogen responsiveness and this response is completely inhibited by administration of the antiestrogen, ICI.

Figure 5. **Gel shift analysis.** The effects of ER expression on PPAR's ability to bind PPRE. Electrophoretic mobility shift assays were performed using nuclear extract from MDA-MB-231 cells not transfected with an expression vector (No ER), and ERα expression vector (ERα) or an ERβ expression vector (ERβ). Lane one contains no nuclear extract. All other lanes contain nuclear extracts and the even lanes lack cold competitor (-) and the odd lanes contained 32-fold excess cold PPRE (+).

Figure 6. **Transient transfection analysis.** Neither the aryl hydrocarbon receptor nor the androgen receptor inhibited PPRE-mediated reporter activation. Cells were transfected with or without the hAhR or the hAR in conjunction with the PPRE-mediated luciferase reporter and the constitutive *Renilla* transfection control reporter. Data is presented as the mean fold induction (n=6) from a representative experiment (±sem).

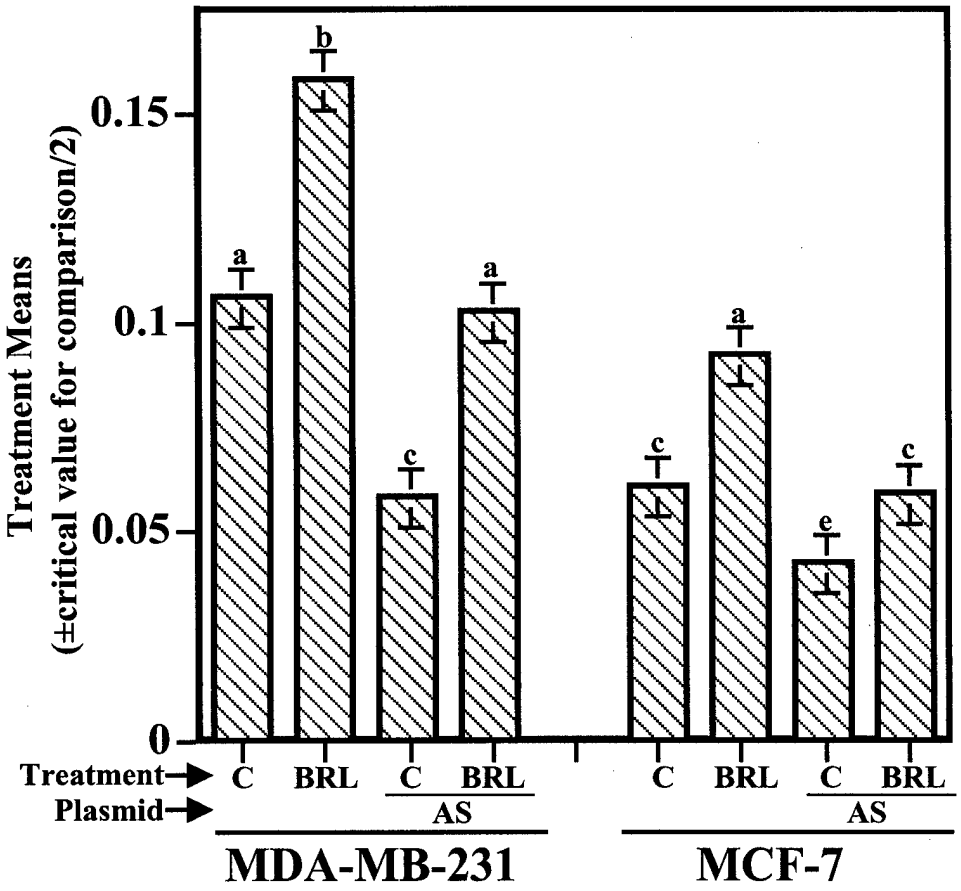
Figure 7. **The effects of ERα mutations on PPAR transactivation.** Cells were transiently transfected with a PPRE-mediated luciferase reporter, the constitutive *Renilla* with or without (No ERα) an expression vector coding either the wild-type ERα (ERα), an ER lacking the AF1 domain, the AF2 domain or the DNA binding domain. Transfection of the wild type ER, the mutant lacking the AF1 domain and the mutant lacking the AF2 domain all repressed PPAR transactivation in unstimulated cells (*) as well as BRL-stimulated (**) cells. The removal of the DNA binding domain abolished inhibition of reporter activation.

Figure 8. Expression of the ER lacking the DNA binding domain inhibits transactivation of ERα in MCF-7 cells. Cells were transfected (plasmid) with a ERE-mediated luciferase reporter and the constitutive *Renilla* with or without (no ER) wild type ERα, the ER mutant lacking the DNA binding domain (DBD^-) or both. Following transfection, cells were then left untreated (Control) or treated with 17β estradiol (E_2). Data are presented as treatment means from 3 separate experiments ± one half the critical value for comparison ($p \leq 0.05$) as determined by ANOVA followed by Tukey's analysis.

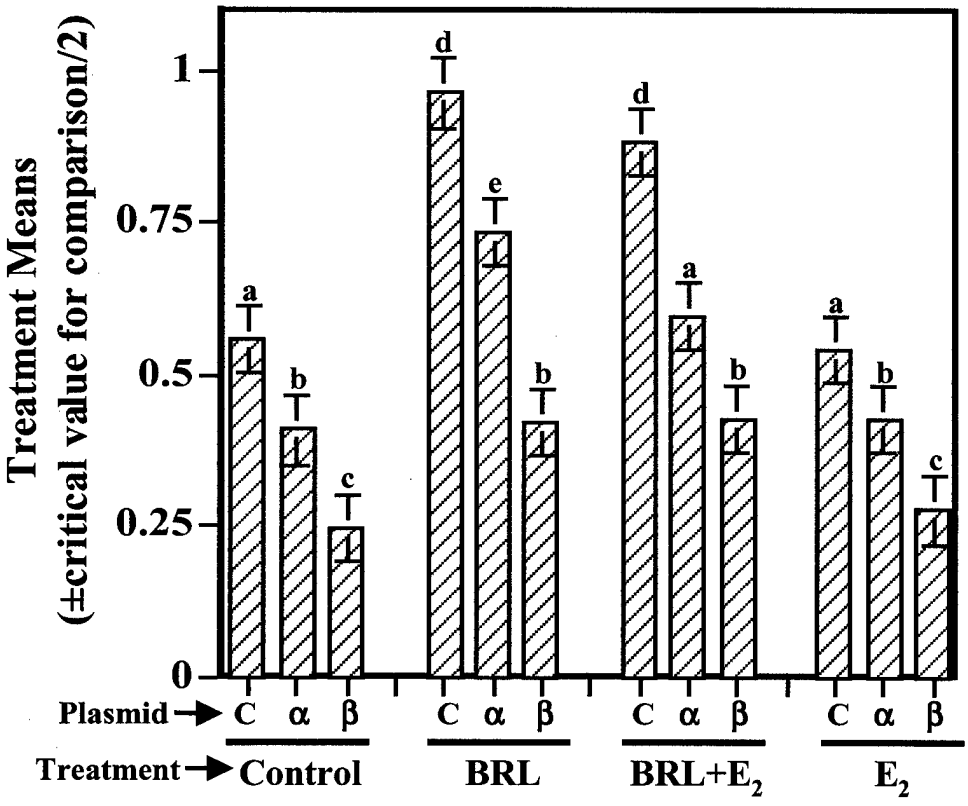
Figure 9. Effects of estrogen treatment on the expression of PPARγ1 in MCF-7 cells. Cells were treated with or without10^{-8} M 17β estradiol for 24, 48 or 72 hrs. Total RNA was isolated and PPARγ expression quantitated by RNase protection assays using a gamma 1-specific probe. Alternatively, cells were treated as described in the presence of 20 μM cycloheximide and PPARγ2 expression measured b RPA.

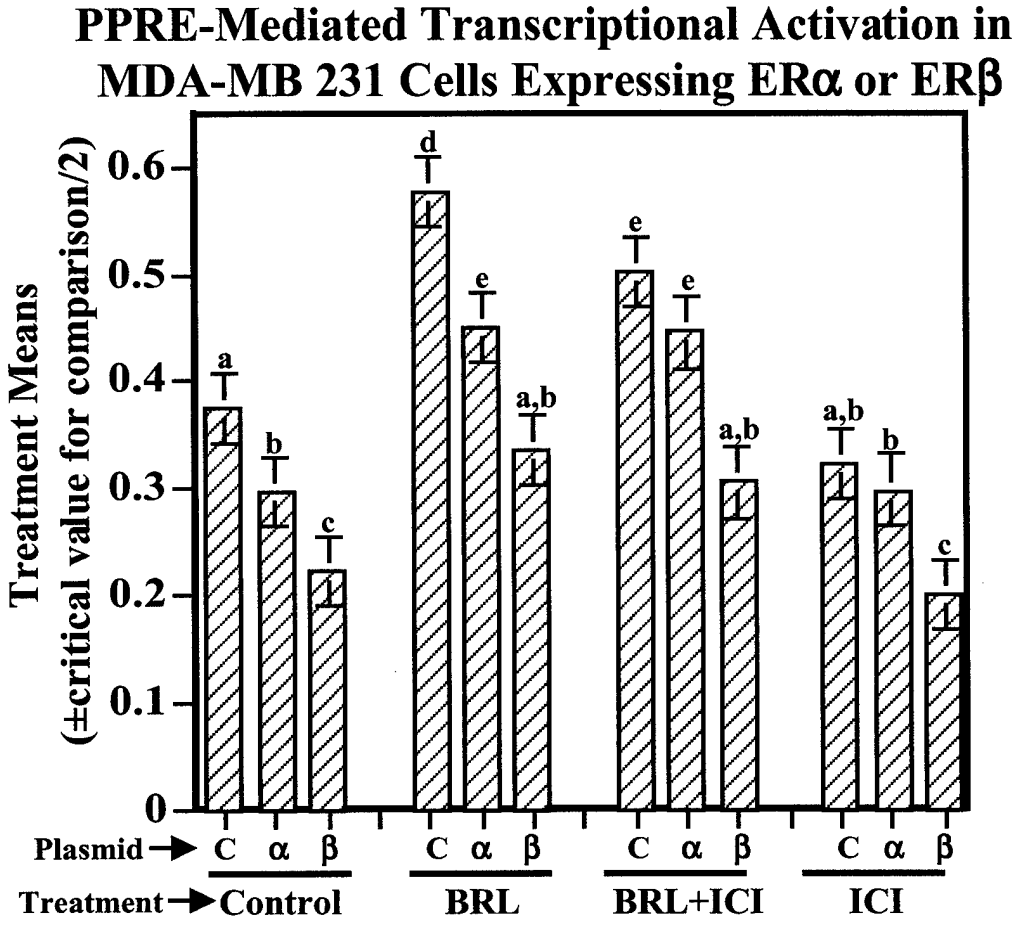
Literature Cited

PPRE-Mediated Transcriptional Activation Is Inhibited by the Expression of PPARγ1 Antisense



PPRE-Mediated Transcriptional Activation in MDA-MB 231 Cells Expressing ERα or ERβ




PPRE-Mediated Transcriptional Activation in MDA-MB 231 Cells Expressing ERα or ERβ
Treatment Means (±critical value for comparison/2)
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0.1
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a,b
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c
Plasmid → C α β C α β C α β C α β
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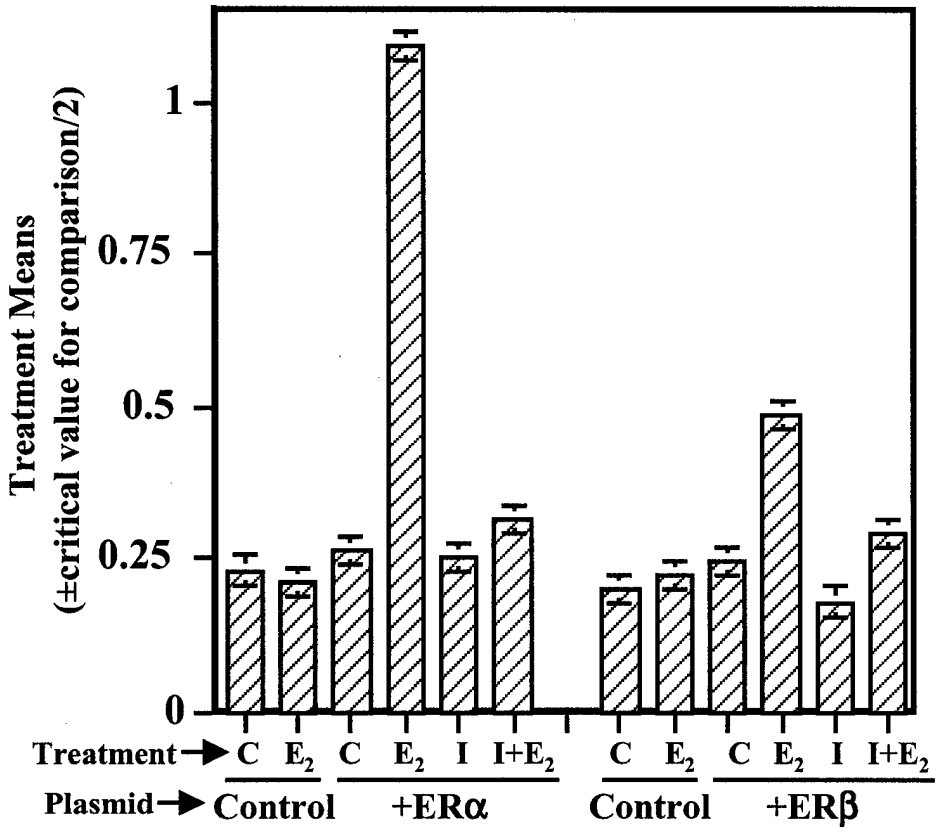
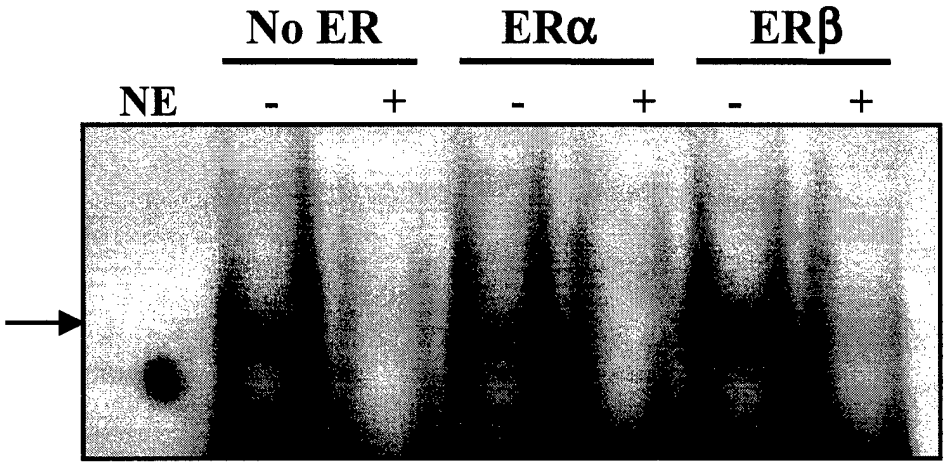
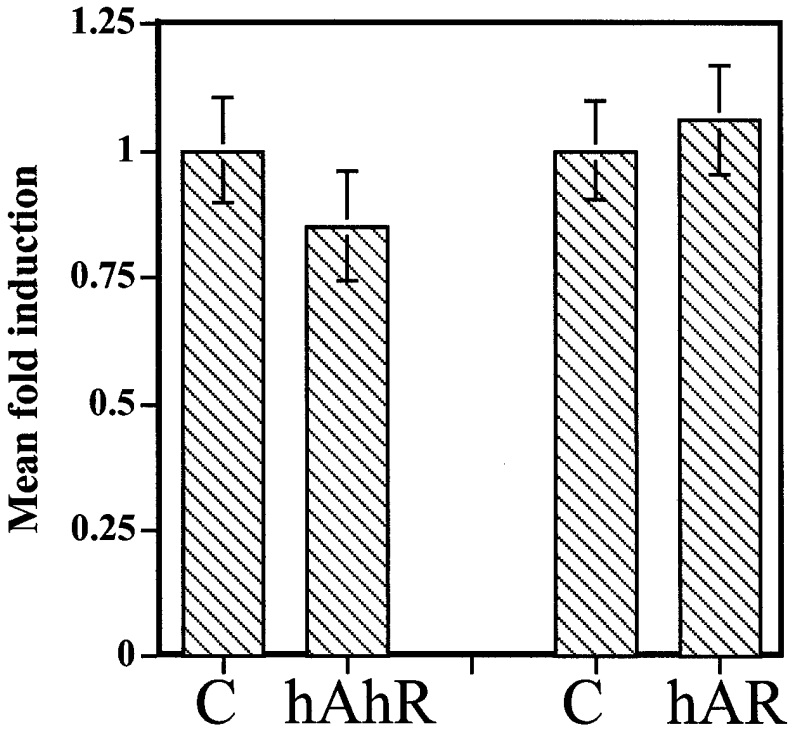
Transfection of MDA-MB-231 Cells with ERα or ERβ Expression Vectors Re-establishes Estrogen Responsiveness.



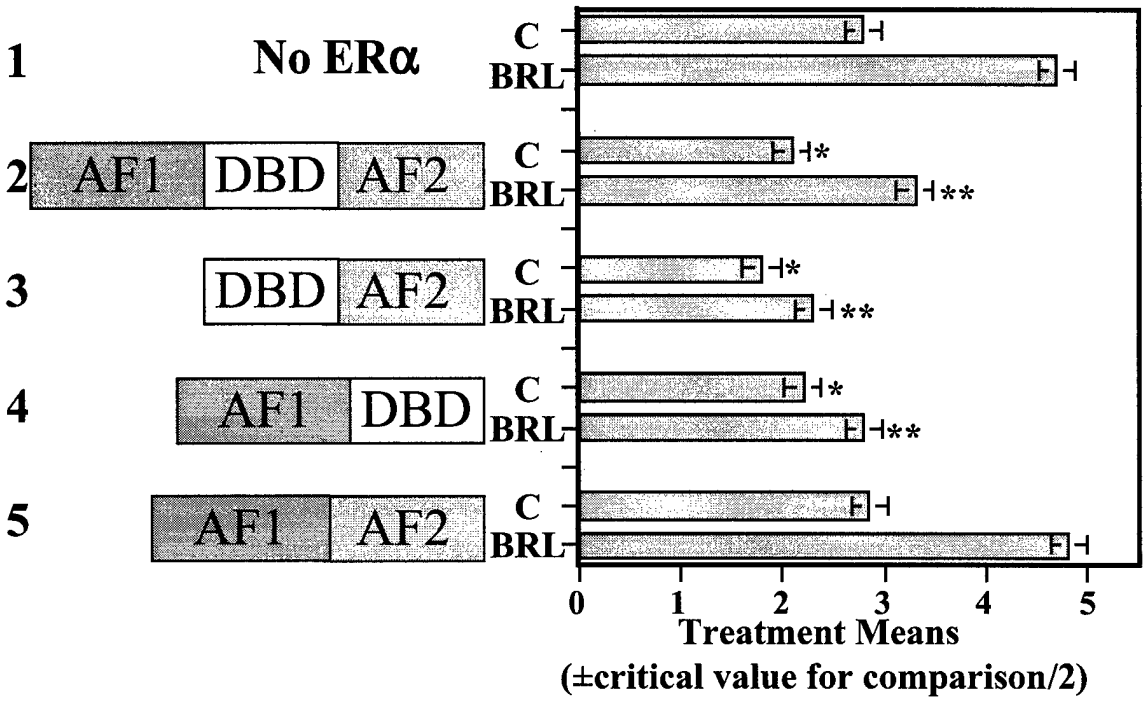
PPRE Binding in MDA-MB-231 cells Expressing ERalpha or ERbeta



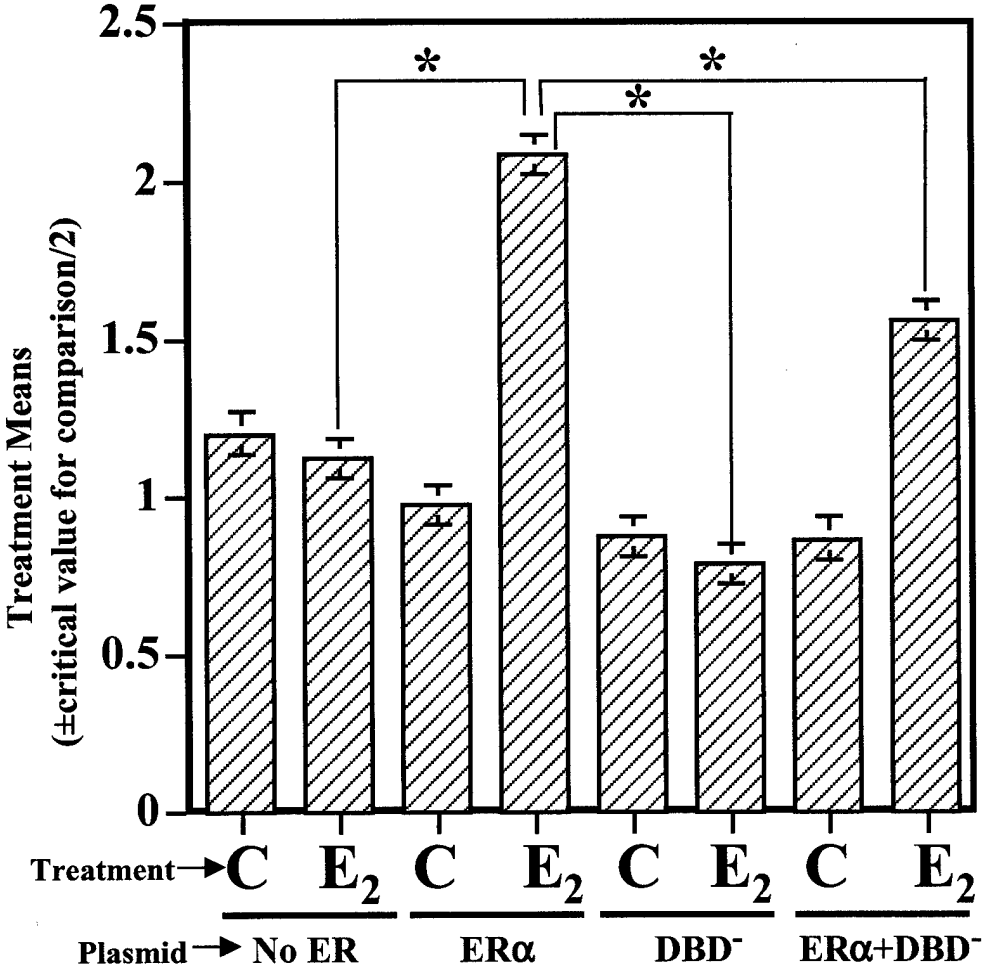
Expression of the Aryl hydrocarbon Receptor or the Androgen Receptor does not Squelch PPRE-mediated Transcriptional Activity in MCF-7 Cells



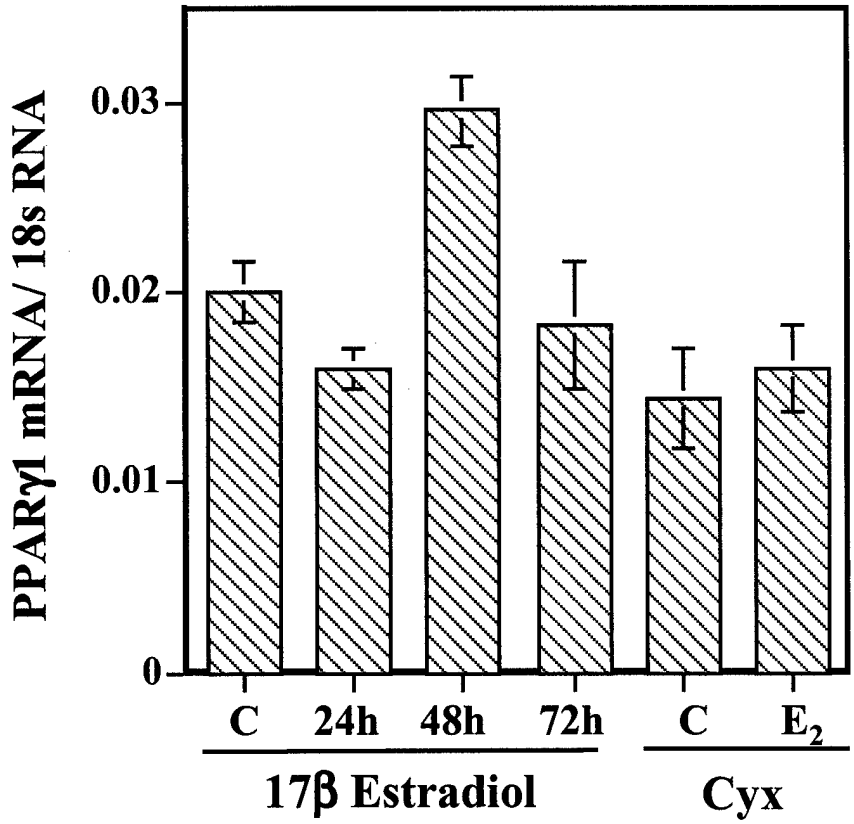
PPRE-Mediated Transcriptional Activation in MDA-MB 231 Cells With and Without Wild-Type ERα and Deletion Mutants



ERE-Mediated Transcriptional Regulation In MDA-MB-231 Cells



Estrogen-mediated Transcriptional Regulation of PPARγ1 Expression in MCF-7 cells



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Differential transcriptional activation of peroxisome proliferator-activated receptor gamma by omega-3 and omega-6 fatty acids in MCF-7 cells

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Abstract

While the role of dietary fats in breast cancer remains controversial, the recent cloning of peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear hormone receptor, from human breast cancer cells lines provides a potential molecular link. Several fatty acids from four classes of dietary fats were tested for their ability to mediate the transcriptional activity of PPARγ in MCF-7 and MDA-MB-231 cells using growth media with minimal serum. Whereas omega-3 fatty acids inhibit transactivation of PPARγ to levels below control, omega-6, monounsaturated and saturated fatty acids stimulate the activity of the transcriptional reporter. These studies indicate that individual fatty acids differentially regulate the transcriptional activity of PPARγ by selectively acting as agonists or antagonists. Furthermore, the transcriptional activation of PPARγ correlates with cell proliferation in MCF-7 cells. Understanding the effects of individual fats on breast cancer cells and PPARγ transactivation could provide important new insights into the epidemiology of breast cancer and the role of dietary fat.

Keywords: PPARγ; Transcription; Breast cancer; Dietary fats; Omega-3 fatty acids; Omega-6 fatty acids

1. Introduction

Breast cancer is the leading cause of the cancer deaths among American women (Menck et al., 1997). In 1997, 180 200 women were diagnosed with breast cancer and approximately 43 900 women died of this disease in the US (Parker et al., 1997). In contrast one in 40 Chinese women will develop breast cancer and this difference cannot be accounted for by genetic factors (Ziegler et al., 1993). Although epidemiological analysis suggests a correlation between high-fat diets and breast cancer in humans (Kelsey and Gammon, 1990; Wynder et al., 1991; Hunter et al., 1996; Greenwald et al., 1997; Rose, 1997; Rohan et al., 1998; Santiago et al., 1998; Simonsen et al., 1998; Snyderwine, 1998), controversy remains. Animal studies, however, have provided convincing evidence of this link and clearly demonstrate a correlation between dietary fats, types of fats ingested and mammary tumors (Karmali et al., 1984; Cohen et al., 1986; Freedman et al., 1990; Cohen et al., 1993; Parkinson et al., 1994; Rose and Hatala, 1994; Rose and Connolly, 1997).

It has been suggested that ω-6 fatty acids, which are high in Western diets, might be associated with higher risk of breast cancer incidences (Rose and Hatala, 1994; Rose, 1997). By contrast, populations whose fat intake is primarily ω-3 fatty acids have a lower incidence of breast cancer (Parkinson et al., 1994) and ω-3 fatty acids inhibit growth and metastatic potential of human cells in animal models (Karmali et al., 1984; Jurkowski

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et al., 1985; Cohen et al., 1993). The results from animal studies suggest that both the amount (Carroll and Braden, 1984; Aksoy et al., 1987; Katz and Boylan, 1989) and type (Braden and Carroll, 1986; Hubbard and Erickson, 1987; Kort et al., 1987; Abou-el-Ela et al., 1989; Cave, 1991; Rose and Connolly, 1993; Rose et al., 1993; Rose and Hatala, 1994; Hubbard et al., 1998) of fats consumed play a role in the susceptibility, growth and metastatic potential of both chemically induced and surgically implanted tumors.

The studies presented in this report were performed to determine whether differential effects of fatty acids on MCF-7 and MDA-MB-231 cells might be mediated by PPARγ. Peroxisome proliferator-activated receptors are members of the nuclear hormone receptor family that play an important regulatory role in adipogenesis and lipid metabolism (Schoonjans et al., 1996; Spiegelman and Flier, 1996). These transcription factors control the expression of genes encoding enzymes in lipid metabolic pathways (Schoonjans et al., 1996; Spiegelman and Flier, 1996). Three genes encoding PPAR have been identified in mammals and are termed PPARα, β/δ and γ (Schoonjans et al., 1996; Spiegelman and Flier, 1996). While the physiological ligand remains unresolved, both synthetic and natural ligands have been reported (Tontonoz et al., 1994; Forman et al., 1995; Kliewer et al., 1995; Schoonjans et al., 1996; Spiegelman and Flier, 1996; Lehmann et al., 1997). Recently we reported that several human breast cancer cell lines express PPARγ and contain a functional response to synthetic peroxisome proliferators (Kilgore et al., 1997). This finding has been confirmed by others in humans (Elstner et al., 1998; Mueller et al., 1998) as well as mice (Gimble et al., 1998). In this report we demonstrate that individual fatty acids selectively function as agonists or antagonists of PPARγ in MCF-7 and MDA-MB-231 cells and the activation of PPARγ correlates with an increase in cell proliferation in MCF-7 cells. This model enables us to begin to examine the molecular mechanism whereby individual components from a complex diet might alter growth and development of breast cancer.

2. Materials and methods

2.1. Chemicals

All fatty acids, Calcium chloride, HeBS reagent chemicals (NaCl, KCl, $Na_2HPO_4.2H_20$), Me_2SO, indomethacin and Bovine Serum Albumin (BSA) were purchased from Sigma (St. Louis, MO). Concentrated stocks of free fatty acids including the monounsaturated fatty acids, (MUFAs) and the polyunsaturated fatty acids (PUFAs) were prepared either in EtOH (Apper Alcohol, Kentucky) or Me_2SO and final concentrations were made by dilutions with the culture medium. Ly 171,883 (Eli Lilly, Indianapolis, IN) was dissolved in EtOH and the final concentrations of EtOH and Me_2SO in all conditions was 0.1%.

2.2. Cell culture

MCF-7 and MDA-MB-231 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in the Dulbecco's modified Eagle's medium (DMEM) or improved MEM (IMEM), respectively (GibCo) containing 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. Cells were gradually adapted to TCH® serum replacement medium (Celox Laboratories, Inc., St. Paul, MN) supplemented with 0.5% FBS (HyClone, Logan, Utah) for MCF-7 or without serum for MDA-MB-231 cells. All media lacked phenol red. Cells were routinely grown in T-75 flasks (Corning) and transferred to 6-well plates (Corning) 2 days prior to transfection. Cells were transfected at $\cong$40–50% confluence and not allowed to go through more than 20 passages to minimize genetic drift inherent in culture system.

2.3. Reporter plasmid construction and transient transfection assays

The DR1-SV40-LUC, was constructed by annealing and ligating complementary 27 bp oligonucleotides containing single copy of underlined DR1 response element (5′-CGCGTGACCAGGTCAAAGGTCA CGTTC) into the unique *Mlu*1-*Xho*1 site of the pGL3-Promoter vector (Promega). The 3 × PPRE-TK-luciferase construct was described previously (Kliewer et al., 1992). MCF-7 and MDA-MB-231 cells were transfected using the calcium phosphate method (Ausbel et al., 1994). Each well received 10 μg 3 × PPRE-luciferase plasmid DNA driven by a minimal TK promoter and 0.33 μg β-galactosidase containing plasmid, constitutively driven by the CMV promoter. Following treatments, cells were lysed in 200 μl lysis buffer and treated according to manufacturer's instructions (Analytical Luminescence Laboratory). Luciferase activity from 40 μl of lysate was measured for 10 s (ALL) and 15 μl lysate was used to measure β-galactosidase activity according to the Galacto-Light instructions (Tropix) for 5 s on a Monolight 2010 (ALL). The relative luminescent values for each well were derived by dividing the inducible luciferase values by the constitutive β-galactosidase values. Stock solutions of omega-3 PUFA, omega-6 PUFA and monounsaturated fatty acids were made in absolute ethanol, and the working solutions were made by dilutions in TCH medium. Stock solutions of saturated fatty acids were prepared in Me_2SO (DMSO) and the working solutions were made in TCH medium containing BSA at a final con-

centration of 0.2%. Each set of treatments was performed in triplicate in three or more independent experiments. Statistical analysis is a randomized block design. Treatment means were compared pairwise using Tukey's multiple comparison analysis and confidence limits were set at $P \leq 0.05$. Data are plotted as the treatment means and the error bars are plus and minus one half of the critical value for comparison, thus error bars that do not overlap represent statistically significant differences.

2.4. Cell proliferation assay

Proliferation was determined by measuring the incorporation of tritiated thymidine into cellular DNA. Thirty hours after treatment 1.0 μCi [^{3}H] thymidine was added to each well and cells incubated for an additional 6 h. Each well was washed four times in PBS and the adherent cells were lysed in 500 μl of 1 × trypsin-EDTA and 500 μl of a lysing solution (2 N NaOH, 6.82 mM, *N*-lauroyl sarcosine and 10 μM EDTA). Lysates were individually transferred into 15 ml centrifuge tubes (Fisher) containing 100 μl of 0.5% Phenol red (Sigma) and 150 μl of formaldehyde, 37% w/w (Fisher). Samples were adjusted to a pH of 7.4 as determined by colormetric analysis against a known standard using HCl and NaOH. Samples from each culture well were passed through a 0.45-μm filter (Millipore) using a 12-filter Millipore manifold. Filters were air-dried, placed in vials with 8 ml of Scintiverse scintillation fluid and counted on a Beckman LS6500. Each set of treatments were performed in replicates of five in three independent experiments. The experimental design is a randomized block design. Treatment means were compared pairwise using Tukey's multiple comparison analysis and confidence limits were set at $P \leq 0.05$. Data are plotted as the treatment means and the error bars are plus and minus one half of the critical value for comparison, thus error bars that do not overlap represent statistically significant differences.

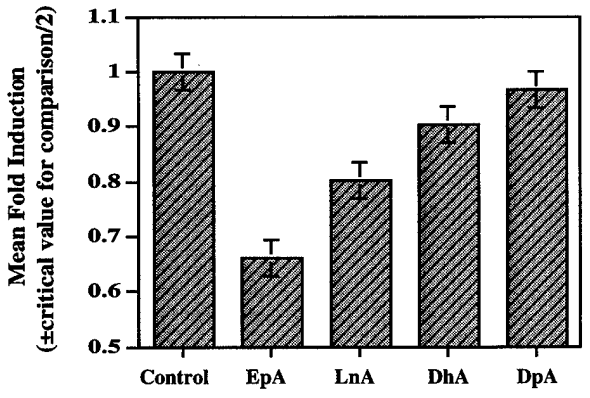

Fig. 1. Effects of omega-3 fatty acids on the transcriptional activity of PPARγ in MCF-7 cells. Cells were transiently transfected with reporter plasmid and treated with vehicle alone (control), eicosapentaenoic acid (EPA), linolenic acid (LnA), eocosahexaenoic acid (DhA) or docosapentaenoic acid (DpA) for 18–24 h. Following ANOVA, data are plotted as the treatment means (± one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($P \leq 0.05$).

3. Results

To determine whether PPARγ activated the DR-1 and 3 × PPRE constructs, both were tested in MCF-7 cells. The synthetic peroxisome proliferator LY 171 883 stimulated reporter activity in cells transfected with either the PPRE or the DR-1 equally (data not shown). Furthermore, the null vectors lacking the DR-1 or the three PPREs were unresponsive indicating the cloned response elements mediated these effects (data not shown).

Next we examined the ability of individual fatty acids to function as ligands and mediate the transcriptional regulation of PPARγ. MCF-7 cells were transiently transfected with the 3 × PPRE-TK-luciferase and treated individually with omega-3 PUFA, omega-6 PUFA, monounsaturated fatty acids or saturated fatty acids. In all cases, the fatty acid concentrations used in these studies were those shown to be maximally effective in this culture system. This was determined over a range of fatty acid concentrations. Of the omega-3 PUFAs, linolenic acid (LnA, C18:3ω3), eicosapentaenoic acid (EpA, C20:5ω3), docosahexaenoic acid (DhA, C22:6ω3) and docosapentaenoic acid (DpA, C22:5ω3) were tested. The results in Fig. 1 demonstrate that omega-3 PUFA inhibit the transcriptional activity of PPARγ to levels below control. The strongest inhibition was observed by EPA (100 μM), which inhibited reporter activity to 66% ($P \leq 0.001$) relative to control. Linolenic acid resulted in 80% reporter activity at 10 nM ($P \leq 0.002$) relative to control. DhA also inhibited PPARγ activity to 89% of control at 10 μM concentration ($P \leq 0.02$). By contrast, DpA did not inhibit the transcriptional activity of PPARγ. Similar results were obtained in another set of experiments in which MCF-7 cells were transfected with DR1-SV40-LUC reporter construct and treated with omega-3 PUFAs (data not shown).

Three omega-6 PUFAs, linoleic acid (LaA, C18:2ω6), arachidonic acid (ArA, C20:4ω6) and γ -linolenic acid, (γ-LnA, C18:3ω6) were tested (Fig. 2). Gamma-Linolenic acid (200 μM) stimulated reporter activity 1.63-fold induction ($P \leq 0.005$) while linoleic acid (250 μM) resulted in a 1.57-fold induction ($P \leq 0.005$) and arachidonic acid (250 μM) produced a 1.52-fold induction ($P \leq 0.005$). Similar results were seen in MDA-MB-231 cells where linoleic acid (250 μM) significantly inducted reporter activity of the 3 × PPRE-luciferase reporter 4.2-fold over control ($P \leq 0.001$, data not shown).

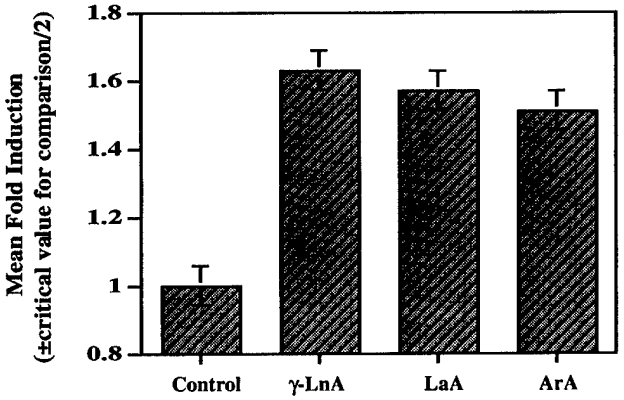

Fig. 2. Effects of omega-6 fatty acids on the transcriptional activity of PPARγ in MCF-7 cells. Cells were transfected with the reporter plasmid and treated with vehicle alone (control), or linoleic acid (LaA), arachidonic acid (ArA) or γ-linolenic acid (γ-LnA), for 18–24 h. Following ANOVA, data are plotted as the treatment means (± one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($P \leq 0.05$).

Fig. 3 demonstrates the effect of two monounsaturated fatty acids (MUFAs), oleic acid (C18:1ω9) and petroselinic acid (C18:1ω12), on PPRE-mediated reporter activity in MCF-7 cells. Oleic acid (350 μM) increased reporter activity 1.24-fold ($P \leq 0.05$) whereas petroselinic acid (150 μM) stimulated reporter activity 1.85-fold ($P \leq 0.001$).

Five saturated fatty acids with increasing chain lengths, caprylic (C10:0), palmitic (C16:0), stearic (C18:0), arachidonic (C20:0) and lignoceric acid (C24:0) were tested (Fig. 4). Both caprylic (10 μM) and palmitic acid (10 μM) weakly stimulated reporter activity 1.15-fold ($P \leq 0.02$) and 1.11-fold, respectively. Lignoceric acid treatment (50 μM) stimulated reporter activity 1.25 over control ($P \leq 0.05$). A 1.2-fold induction ($P \leq 0.05$) was seen with arachidonic acid treatment (100 μM) and stearic acid (250 μM) increased reporter activity 1.4-fold ($P \leq 0.002$).

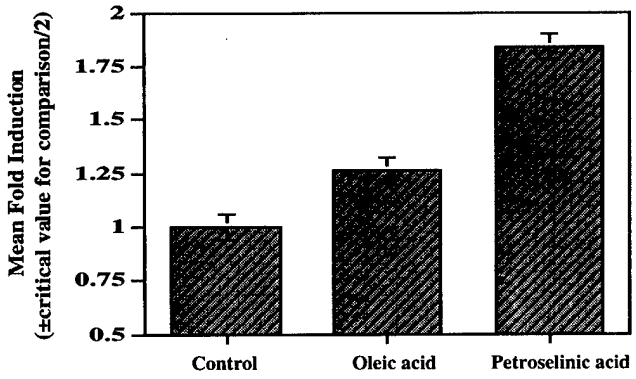

Fig. 3. Effects of monounsaturated fatty acids on the transcriptional activity of PPARγ in MCF-7 cells. Following transfection, cells were treated for 18–24 h with vehicle alone (control), oleic or petroselinic. Following ANOVA, data are plotted as the treatment means (± one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($P \leq 0.05$).

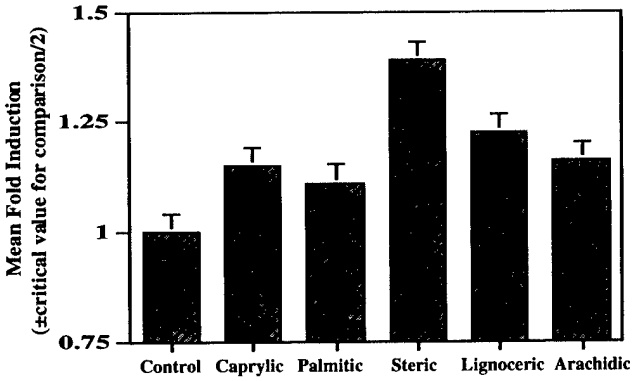

Fig. 4. Effects of saturated fatty acids on the transcriptional activity of PPARγ MCF-7 cells. MCF-7 cells were transfected with the reporter plasmid and treated with vehicle alone (control), caprylic, palmitic, stearic, arachidic or lignoceric acid, as described, for 18–24 h. Following ANOVA, data are plotted as the treatment means (± one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($P \leq 0.05$).

Using the same concentrations reported in the transcriptional assays, the effects of linoleic and linolenic acids were examined for their ability to induce proliferation (Fig. 5). Estrogen, in the form of 17β-estradiol, a known mitogen in MCF-7 cells, significantly increased cell proliferation as did linoleic acids ($P \leq 0.001$ and $P \leq 0.005$, respectively). By contrast, linolenic acid significantly inhibited cell proliferation to levels below control ($P \leq 0.01$).

4. Discussion

In an attempt to further clarify the role of individual dietary components on the physiology of human breast cancer cells, we have tested a variety of fatty acids for

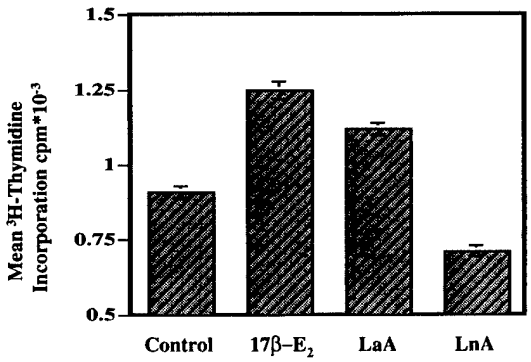

Fig. 5. Effects of 17β-estradiol (E_2), linoleic and linolenic acid on MCF-7 cell proliferation. Cells were plated at equal densities and treated for 30 h with physiological concentrations of estradiol or of fatty acids at the same concentrations used in transfection experiments. Each well received ^{3}[H]thymidine plus treatments for an additional 6 h. Following ANOVA, data are plotted as the treatment means (± one half of the critical value for comparison). Error bars that do not overlap represent statistically significant differences ($P \leq 0.05$).

their ability to modulate the transcriptional activity of PPARγ. Three important observations have been made as a consequence of these studies. First, individual fatty acids appear to selectively function as agonists or antagonists of PPARγ in MCF-7 cells. Although variability exists between individual fatty acids within a class, clear differences exist between the classes of fatty acids themselves. These data suggest that transactivation of PPARγ may be one mechanism whereby individual fatty acids can mediate cell-specific function by modulating gene expression. The fact that reporter activity was also stimulated in MDA-MB-231 cells with linoleic acid indicates this is not a response specific to MCF-7 cells and may be more generally applicable to other breast cancer cells. Secondly, while we have previously demonstrated that MCF-7 cells express high levels of mRNA, these data suggest that PPARγ is constitutively transactivated in MCF-7 cells. This is supported by the observation that ω-3 fatty acids such as linolenic acid inhibits the activity of the transcriptional reporter to levels below that of control. This suggests that linolenic acid can compete with some endogenous ligand thus inhibiting basal activity. Thirdly, linoleic acid, an agonist of PPARγ function, increases the rate of cell proliferation while linolenic acid treatment, a PPARγ antagonist, reduces proliferation. This is in contrast to troglitazone, a synthetic agonist of PPARγ, which has been reported to inhibit proliferation of 21MT cells (Mueller et al., 1998) and induce apoptosis when used in combination with all-*trans*-retinoic acid in MCF-7 cells (Elstner et al., 1998). Evans and co-workers, however, have demonstrated that activators of PPARγ increase the incidence of colorectal tumors and polyp formation indicating that transactivation of PPARγ may indeed result in tumor formation (Saez et al., 1998). In support of our proliferation studies animals fed linolenic acid have fewer tumors and a reduced metastatic potential when compared to animals maintained on diets rich in linoleic acid (Cave, 1991; Rose et al., 1994). The differences between the in vitro and in vivo data could be a function of cell-specific responses or due to differences in the ligand-receptor interactions. Although these data do not prove a cause and effect relationship between transactivation of PPARγ and the control of cell cycle, the mechanism of this effect in MCF-7 cells clearly warrants further investigation.

In NIH 3T3 cells, a mouse adipose stromal cell line, transfection with PPARγ is both necessary and sufficient to set in motion the differentiation into an adipocyte. The presence of PPARγ in human breast cancer cells has led to the speculation that it's transactivation could be used therapeutically to induce the re-differentiation of malignant cells into a benign state (Mueller et al., 1998). Here we report that MCF-7 cells express PPARγ and the receptor exists in some continuous state of transcriptional activation, yet these cells do not differentiate into adipocytes. This suggests that the transactivation of PPARγ in MCF-7 cells sets in motion a program unique from that seen in adipose stromal cells. Clearly, it will be important to examine the genes regulated by PPARγ in human breast cancer cells to understand the functional significance of these observations. Additionally, further studies will be necessary to determine the molecular mechanisms of tissue-specific responses.

A question critical to determining the role PPARγ plays in human breast cancer is to assess its expression in normal ductile epithelia as well as primary and metastatic tumors. Gimble et al. (1998) reported that PPARγ is expressed in ductile epithelia of virgin mice and rats. However, PPARγ is not expressed during lactation nor in mammary tumors induced by 7,12-dimethylbenz(a)anthracene (Gimble et al., 1998). Furthermore, NmuMG, a normal mouse epithelial line, expresses PPARγ but its expression is not inducible by peroxisome proliferators. In contrast to the rodent model, PPARγ is expressed in several human cancer cell lines including T-47-D, MDA-MB-231, SK-BR3, ZR-75-1 and BT-20 cells (Kilgore et al., 1997; Mueller et al., 1998) and a functional response to peroxisome proliferators has been demonstrated in MCF-7 (Kilgore et al., 1997) and 21MT cells (Mueller et al., 1998). Finally, Spiegelman and coworkers have shown that PPARγ is expressed in both primary breast tumors and in lung sections of patients with metastatic tumors (Mueller et al., 1998). The expression of PPARγ in both benign and malignant cells leaves open the possibility that peroxisome proliferators could play a role in both normal and cancerous tissue.

The role of lipid in breast disease has been the subject of intense debate and, more recently, intensive investigation. The identification of a functional response to peroxisome proliferators in a wide variety of breast cancer cell lines and the ability of fatty acids to selectively mediate the transactional activity of PPARγ lends further support for a direct role of dietary fatty acids in human breast cancer. Clearly, the functional significance of these findings will require further investigation.

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